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(54) Title: A PROTEIN PREPARATION THAT MEDIATES Ca^{2+}-DEPENDENT TRANSBILAYER MOVEMENT OF PLASMA MEMBRANE PHOSPHOLIPID AND INHIBITORS THEREOF

(57) Abstract

A protein preparation that mediates Ca^{2+}-dependent transbilayer movement of phospholipids is disclosed. A recombinantly engineered DNA sequence encoding the protein, an inhibitor of the protein activity, genetically engineered cells with altered protein activity, and therapeutic methods are also disclosed.

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A PROTEIN PREPARATION THAT MEDIATES Ca^{2+}-DEPENDENT TRANSBILAYER MOVEMENT OF PLASMA MEMBRANE PHOSPHOLIPID AND INHIBITORS THEREOF

United States Government may have commercial rights under Grant R01 HL36946 from Heart, Lung, & Blood Institue, National Institutes of Health.

BACKGROUND OF THE INVENTION

The exposure of phosphatidylserine (**PS**) and other aminophospholipids (**aminoPL**) on the surface of activated or injured blood cells and endothelium is thought to play a key role in the initiation and regulation of blood coagulation. De novo surface exposure of aminophospholipids has also been implicated in the activation of both complement and coagulation systems after tissue injury, and in removal of injured or apoptotic cells by the reticuloendothelial system. Although migration of these phospholipids (**PL**) from inner-to-outer plasma membrane leaflets is known to be triggered by elevated intracellular [Ca^{2+}] ([Ca^{2+}]$_i$) and to be associated with vesicular blebbing of the cell surface, little is known about the cellular constituents that participate in this process.

Role of cell surface PS in coagulation. Several enzyme complexes of the coagulation cascade require assembly on a receptive membrane surface for full expression of catalytic activity (K. G. Mann, *et al.*, *Annu. Rev. Biochem.* 57:915-956, 1988; S. Krishnaswamy, *et al.*, *J. Biol. Chem.* 267:26110-26120, 1992; P. B. Tracy, *Semin. Thromb. Hemost.* 14:227-233, 1988). In the case of the tenase (FVIIIaFIXa) and prothrombinase (FVaFXa) complexes, this surface catalytic function of the plasma membrane is not normally expressed by quiescent cells, but is rapidly induced upon cell activation (in platelets) or upon cell injury (in platelets, endothelium and other cells) (E. M. Bevers, *et al.*, *Blood Rev.* 5:146-154, 1991; J. Rosing, *et al.*, *Blood* 65:319-332, 1985; E. M. Bevers, *et al.*, *Eur. J. Biochem.* 122:429-436, 1982; E. M. Bevers, *et al.*, *Biochim.*

Biophys. Acta 736:57-66, 1983; T. Wiedmer, *et al.*, *Blood* 68:875-880, 1986). Although specific cell surface protein receptors for FVa and FVIIIa have been postulated, these factors show specific avidity for PS-containing liposomes, and in cell-free systems, this lipid alone can support the catalytic function of the prothrombinase and tenase enzymes (J. Rosing, *et al.*, *supra*, 1985; M. E. Jones, *et al.*, *Thromb. Res.* 39:711-724, 1985; G. E. Gilbert, *et al.*, *Biochemistry* 32:9577-9585, 1993; G. E. Gilbert, *et al.*, *J. Biol. Chem.* 265:815-822, 1990; G. E. Gilbert, *et al.*, *J. Biol. Chem.* 267:15861-15868, 1992). We and others have shown that PS rapidly moves to the surface of the plasma membrane upon platelet stimulation, and that this exposure of PS correlates with expression of the platelet's FVa & FVIIIa binding sites and expression of surface catalytic function for tenase and prothrombinase (P. Williamson, *et al.*, *Biochemistry* 31:6355-6360, 1992; F. Bassé, *et al.*, *Biochemistry* 32:2337-2344, 1993; C.-P. Chang, *et al.*, *J. Biol. Chem.* 268:7171-7178, 1993; J. Connor, *et al.*, *Biochim. Biophys. Acta*1025:82-86, 1990; P. Comfurius, *et al.*, *Biochim. Biophys. Acta* 1026:153-160, 1990). Smeets, *et al.*, *Biochim. Biophys. Acta* 1195:281-286, 1994, Williamson, *et al.*, *Biochem.* 34:10448-10455, 1995; Bratton D.L., *J. Biol. Chem.* 269:22517-22523, 1994). Additional evidence that surface-exposed PS provides the physiological receptor site for these enzyme complexes is provided by (1) the capacity of PS-containing liposomes or phosphoserine to compete binding of FVIIIa to activated platelets (G.E. Gilbert, *et al.*, *J. Biol. Chem.* 266:17261-17268, 1991), (2) the capacity of annexin V and other proteins with affinity for membrane PS to mask the FVa and FVIIIa binding sites expressed by activated platelets (P. Thiagarajan, *et al.*, *J. Biol. Chem.* 265:17420-17423, 1990; P. Thiagarajan, *et al.*, *J. Biol. Chem.* 266:24302-24307, 1991; J. Dachary-Prigent, *et al.*, *Blood* 81:2554-2565, 1993; J. Sun, *et al.*, *Thromb. Res.* 69:289-296, 1993); (3) evidence that platelets congenitally deficient in inducible FVa and FVIIIa receptors are also defective in stimulated exposure of plasma membrane PS ("Scott syndrome"; *see below*) (J. P. Miletich, *et*

al., *Blood* 54:1015-1022, 1979; J. Rosing, *et al.*, *Blood* 65:1557-1561, 1985; P. J. Sims, *et al.*, *J. Biol. Chem.* 264:17049-17057, 1989; S. S. Ahmad, *et al.*, *J. Clin. Invest.* 84:824-828, 1989; F. Toti, *et al.*, *Blood* 87:1409-1415, 1996). In addition to the catalytic function PS provides to the prothrombinase and tenase complexes, surface exposed aminophospholipids have been shown to promote the activities of the tissue factor-FVIIa and protein S-activated protein C enzyme complexes of the coagulation system , as well as the activity of the alternative pathway C3-convertase (C3bBb enzyme complex) of the complement system (W. Ruf, *et al.*, *J. Cell. Biol.* 266:2158-2166, 1991; F. J. Walker, *J. Biol. Chem.* 256:11128-11131, 1981; R. H. Wang, *et al.*, *J. Clin. Invest.* 92:1326-1335, 1993; P. F. Neuenschwander, *et al.*, *Biochemistry* 34:13988-13993, 1995).

In addition to the central role that inducible expression of plasma membrane PS is thought to play in the platelet hemostatic response, the surface exposure of PS and phosphatidylethanolamine (PE) in response to membrane injury has been implicated in a variety of thrombotic and inflammatory disorders. For example, repeatedly sickled SS hemoglobin erythrocytes exhibit increased surface exposure of PS, which promotes prothrombinase assembly and accelerates plasma clotting *in vitro*, and may contribute to thrombotic complications that can arise in sickle cell disease (P. F. Franck, *et al.*, *J. Clin. Invest.* 75:183-190, 1985; N. Blumenfeld, *et al.*, *Blood* 77:849-854, 1991). Increased PE exposure on sickled RBCs (and other cells) has also been shown to promote complement activation with resulting accumulation of C3b/C3d and C5b-9 on the cell surface, potential factors contributing to the accelerated clearance and increased fragility of these cells (R. H. Wang, *et al.*, *supra*, 1993). PS exposure secondary to immune injury to the endothelium has also been implicated in the thrombo-embolic complications of hyperacute graft rejection, and PS exposure secondary to C5b-9 accumulation on platelets and red cells has been suggested to contribute to the high risk of venous thrombosis in Paroxysmal Nocturnal Hemoglobinuria (J. L. Platt, *et al.*, *Immunol. Today*

11:450-6; discuss, 1990; A. P. Dalmasso, *Immunopharmacology* 24:149-160, 1992; A. P. Dalmasso, *et al.*, *Am. J. Pathol.* 140:1157-1166, 1992; T. Wiedmer, *et al.*, *Blood* 82:1192-1196, 1993, K. K. Hamilton, *et al.*, *J. Biol. Chem.* 265:3803-3814, 1990; S. P. Kennedy, *et al.*, *Transplantation* 57:1494-1501, 1994)). In the "antiphospholipid syndromes," the interaction of exposed plasma membrane PS and PE with plasma proteins is now generally believed to induce offending antigens (M.D. Smirnov, *et al.*, *J. Clin. Invest.* 95: 309-316, 1995).

Relationship of PS exposure to programmed cell death. Programmed cell death (apoptosis) is now recognized to be central to the selective elimination of mammalian cells during embryogenesis, tissue re-modeling, and in the clonal selection of immune cells (P. D. Allen, *et al.*, *Blood Rev.* 7:63-73, 1993; J. J. Cohen, *Immunol. Today* 14:126-130, 1993). The apoptotic cell undergoes characteristic changes, including elevated $[Ca^{2+}]_i$, altered phospholipid packing, surface exposure of PS, plasma membrane blebbing and vesiculation, cell shrinkage, chromatin condensation, nucleolar desintegration, and at late stages, DNA degradation by Ca^{2+}/Mg^{2+}-dependent endonuclease(s), with characteristic fragmentation into 180 bp multimers ("DNA laddering"). The transcriptional events that initiate apoptosis remain unresolved, but evidence implicates certain proto-oncogenes, including c-myc as activators, and other proto-oncogenes, including bcl-2, as suppressors (P. D. Allen, *et al.*, *supra*, 1993; J. C. Reed, *J. Cell. Biol.* 124:1-6, 1994). In thymocytes and B-lymphocytes, an apoptotic transformation can be induced by dexamethasone (activating glucocorticoid receptors) and by cAMP (protein kinase A pathway) (D. J. McConkey, *et al.*, *J. Immunol.* 145:1227-1230, 1990; N. Kaiser, *et al.*, *Proc. Natl. Acad. Sci. USA* 74:638-642, 1977; J. J. Cohen, *et al.*, *J. Immunol.* 132:38-42, 1984; R. Merino, *et al.*, *EMBO J.* 13:683-691, 1994; M. K. Newell, *et al.*, *Proc. Natl. Acad. Sci. USA* 90:10459-10463, 1993), as well as directly through treatment with Ca^{2+} ionophore (Z.-Q. Ning, *et al.*, *Eur. J. Immunol.* 23:3369-3372, 1993), implicating $[Ca^{2+}]_i$ as a

central mediator of the cellular changes that accompany apoptosis. The similarity of the plasma membrane changes noted for apoptotic cells, to those elicited by elevation of $[Ca^{2+}]_i$ in platelets, erythrocytes, and other cells that do not undergo apoptosis, suggest that the nuclear and plasma membrane changes associated with apoptosis are separate "epiphenomena", reflecting independent and unrelated responses to a coordinate rise in $[Ca^{2+}]_i$.

Diaz, *et al.* (*Blood* 87[7]:2956-2961, 1996) have recently reported the generation of phenotypically aged phosphatidylserine-expressing erythrocytes by dilauroylphosphatidylcholine (DLPC)-induced vesiculation. Red blood cells were artificially vesiculated with DLPC and assessed for alterations in density, membrane lipid asymmetry and propensity to be recognized by macrophages *in vitro* and the reticuloendothelial system *in vivo*. The results suggest that vesiculation contributes to alterations is membrane lipid asymmetry and cell characteristics of the aged red blood cell phenotype.

Role of cell surface PS in clearance by the RE system. There is now accumulating data to suggest that cell-surface PS may contribute to the recognition and clearance of senescent, injured, or apoptotic cells by macrophages and other cells of the reticuloendothelial system (J. Savill, *et al.*, *Immunol. Today* 14:131-136, 1993; V. A. Fadok, *et al.*, *J. Immunol.* 148:2207-2216, 1992; J. Connor, *et al.*, *J. Biol. Chem.* 269:2399-2404, 1994). These experiments demonstrate that (i) macrophages have inducible receptors that stereospecifically bind to PS-containing liposomes and to surface-exposed plasma membrane PS; (ii) selective phagocytosis of apoptotic lymphocytes by stimulated macrophages is observed in the absence of plasma proteins, and this can be inhibited by PS-containing liposomes or by phosphoserine (V. A. Fadok, *et al.*, *supra*, 1992). Consistent with these data, the circulating lifetime of infused PS-containing liposomes is markedly decreased when compared to liposomes devoid of PS, due to rapid hepato-splenic clearance (T. M. Allen, *et al.*,

Proc. Natl. Acad. Sci. USA 85:8067-8071, 1988). Similarly, increased exposure of plasma membrane PS during *in vitro* storage of platelet concentrates may contribute to an accelerated clearance of these cells after transfusion (A. P. Bode, *et al.*, *Thromb. Res.* 39:49-61, 1985; A. P. Bode, *et al.*, *J. Lab. Clin. Med.* 113:94-102, 1989; A. P. Bode, *et al.*, *Blood* 77:887-895, 1991; D. Geldwerth, *et al.*, *J. Clin. Invest.* 92:308-314, 1993; P. Gaffet, *et al.*, *Eur. J. Biochem.* 222:1033-1040, 1994; E. M. Bruckheimer, *et al.*, *J. Leukoc. Biol.* 59, 784-788, 1996; C. Diaz, *et al*, *supra*, 1996). This possibility is underscored by recent reports documenting increased PS exposure in platelets and red cells during *in vitro* storage. Evidence that PS exposed on the surface of tumor cells promotes adherence and cytolysis by inflammatory macrophages has also been reported (J. Connor, *et al.*, *Proc. Natl. Acad. Sci. USA* 86:3184-3188, 1989).

Regulation of the transmembrane distribution of PS. It is now well established that phospholipids are normally asymmetrically distributed within the plasma membrane of all blood cells, vascular endothelium, and other cells: the aminophospholipids (including phosphatidylserine (**PS**) and phosphatidylethanolamine (**PE**)) reside almost exclusively in the inner membrane leaflet, whereas the outer leaflet is enriched in neutral polar phospholipids, including phosphatidylcholine (**PC**) and sphingomyelin (B. Roelofsen, *Infection* 19:S206-S209, 1992; A. J. Schroit, *et al.*, *Biochim. Biophys. Acta* 1071:313-329, 1991; P. F. Devaux, *Biochemistry* 30:1163-1173, 1991). It is well-recognized that the transmembrane orientation of plasma membrane PL is central to the regulation of surface-localized enzyme reactions of both complement and coagulation systems and to the recognition and phagocytic clearance of injured, aged or apoptotic cells. It is also now generally accepted that the maintenance of PL asymmetry arises through the activity of a specific transmembrane PL "flippase" with specificity for aminoPL. This aminoPL translocase (**APT**) has been shown to selectively and vectorially transport PS (> PE), but not neutral PL such

as PC, from outer to inner leaflets of the plasma membrane in a process that is dependent on both Mg^{2+} and ATP, inhibited by fluoride, o-vanadate or increased $[Ca^{2+}]_c$, and inactivated by N-ethylmaleimide (**NEM**) or pyridyldithioethylamine (**PDA**)(M. Bitbol, *et al.*, *Biochim. Biophys. Acta* 904:268-282, 1987; M. Seigneuret, *et al.*, *Proc. Natl. Acad. Sci. USA* 81:3751-3755, 1984; J. Connor, *et al.*, *Biochemistry* 26:5099-5105, 1987; P. F. Devaux, *et al.*, *Phys. Lipids* 73:107-120, 1994; A. Zachowski, *et al.*, *Biochemistry* 25:2585-2590, 1986; C. Diaz, *et al.*, *supra*, 1996). In addition to plasma membrane, APT activity has also been identified in the membranes of secretory vesicles and synaptosomes (A. Zachowski, *et al.*, *Nature* 340:75-76, 1989). The K_m for ATP is approximately 1 mM, and it has been estimated that one molecule of ATP is hydrolyzed for each aminoPL transported (Z. Beleznay, *et al.*, *Biochemistry* 32:3146-3152, 1993). Two candidate proteins have been proposed to function as APT: the Rh antigen protein, and a 110-120 kDa Mg^{2+}-ATPase. Schroit and coworkers (A. J. Schroit, *et al.*, *Biochemistry* 29:10303-10306, 1990) originally proposed that a 32 kDa PS-binding RBC membrane protein that precipitated with antibody to Rh was the erythrocyte APT. Subsequently it was shown that Rh_{null} cells deficient in Rh antigen nevertheless exhibit normal APT activity, and very recently, the 32 kDa PS-binding protein that co-precipitates with Rh protein was identified as stomatin, and it was shown that its interaction with PS was not specific for the aminoPL headgroup. This implies that neither stomatin, nor, the Rh protein can provide APT function (J. Desneves, *et al.*, *Biochem. Biophys. Res. Commun.* 224:108-114, 1996). The observed similarity in cation, ATP- and PS-dependence of cellular APT activity to a partially purified Mg^{2+}-dependent ATPase from RBC led Devaux and associates, and later Daleke, to suggest that APT is a specific Mg^{2+}-ATPase (A. Zachowski, *et al.supra*, 1989; G. Morrot, *et al.*, *FEBS Lett.* 266:29-32; D. L. Daleke, *et al.*, *Ann. NY Acad. Sci.* 671:468-470, 1992; M. L. Zimmerman, *et al.*, *Biochemistry* 32:12257-12263, 1993). Consistent with this premise, Auland (Auland, *et al.*, *Proc. Natl. Acad. Sci* 91:10938-10942, 1994) demonstrated PS-specific

transport in proteoliposomes reconstituted with an unidentified Mg^{2+}-ATPase isolated from RBC. Recently, an ATPase II from bovine chromaffine granules has been cloned and sequenced, and evidence has been presented that this enzyme may exhibit aminoPL translocase activity (X. J. Tang, *et al.*, *Science* 272:1495-1497, 1996).

Ca^{2+} and the collapse of phospholipid asymmetry

Whereas the rate of spontaneous flip/flop of PL between membrane leaflets is normally quite slow, a substantial rise in $[Ca^{2+}]_c$ resulting from agonist-induced activation, programmed cell death, or, secondary to immune injury, initiates rapid transbilayer migration of all plasma membrane PL with net movement of aminoPL to the outer leaflet, collapsing the normal asymmetric distribution (P. Williamson, *et al.*, *Biochemistry* 31:6355-6360, 1992); F. Bassé, *et al.*, *Biochemistry* 32:2337-2344, 1993; C.-P. Chang, et al., *supra*, 1993; P. Comfurius, *et al. Biochim. Biophys. Acta* 1026:153-160 1990; A. J. Schroit, *supra*, 1991; P. Devaux, *Biochemistry* 30:1163-1173, 1991; J. Connor, *et al.*, *J. Biol. Chem.* 267:19412-19417, 1992). Four different mechanisms have been proposed to account for this induced "scrambling" of plasma membrane PL with net egress of aminoPL to cell surfaces (A. J. Schroit, *supra*, 1991; P. Devaux, *supra*, 1992; P. Devaux, *supra*, 1991; R. F. A. Zwaal, *et al.*, *Biochim. Biophys. Acta* 1180:1-8, 1992): (i) spontaneous collapse of PL asymmetry due to inactivation of vectorial transport by plasma membrane APT; (ii) random scrambling due to transient formation of non-bilayer (H_{II}-phase) PL domains upon Ca^{2+}-induced blebbing of plasma membrane vesicles; (iii) direct effects of Ca^{2+} on topology and distribution of anionic PLs; (iv) response of a Ca^{2+}-sensitive protein(s) that facilitates PL transfer between membrane leaflets.

(i) Spontaneous collapse of PL asymmetry. APT is inhibited at elevated $[Ca^{2+}]_c$, raising the possibility that the concomitant egress of aminoPL to the cell surface simply reflects spontaneous back-leak ("flop") of the PL distribution that is constitutively maintained by APT (P. Williamson, *et al.*,

supra, 1992; P. F. Devaux, *supra*, 1992; P. Devaux, *supra*, 1991). Nevertheless, inhibition of APT--either by depletion of cellular ATP, or by incubation with fluoride, o-vanadate or NEM--does not in itself cause accelerated transbilayer PL migration or significant cell-surface PS exposure, as long as normally low $[Ca^{2+}]_c$ is maintained (E. M. Bevers, *et al.*, *supra*, 1991; P. Comfurius, *et al. supra*, 1990; B. Verhoven, *et al.*, *Biochim. Biophys. Acta* 1104:15-23, 1992; J. Connor, *et al. Biochemistry* 29:37-43, 1990). Conversely, cells genetically deficient in PL scramblase show normal APT activity. Thus it appears that the spontaneous transbilayer migration of plasma membrane PL is inherently very slow at the normal low $[Ca^{2+}]_c$, whereas entry of Ca^{2+} into the cytosol specifically induces rapid movement of PL between plasma membrane leaflets. Whereas the inherent rate of transbilayer migration of PL cannot account for the rapid scrambling observed at elevated $[Ca^{2+}]_c$, it is conceivable that interaction of $[Ca^{2+}]_c$ with APT induces a conformational change that not only inactivates the ATP-dependent inward translocation of aminoPL, but also facilitates selective flop of PS & PE to the outer leaflet. In this context, Bienvenue and associates (Bassé, *et al*, *supra*, 1993; Gaffet, *et al.*, *Biochemistry* 34:6762-6769, 1995) have reported evidence for transient vectorial egress of PS upon elevation of $[Ca^{2+}]_c$ in platelet, whereas data from others suggest bidirectional and non-selective transbilayer scrambling of all plasma membrane PL (including PC which is not flipped by APT) at elevated $[Ca^{2+}]_c$ (P. Williamson, *et al.*, *supra*, 1992; P. Williamson, et al., Biochemistry 34:10448-10455, 1995; E. F. Smeets, et al., Biochim. Biophys. Acta Bio-Membr. 1195:281-286, 1994; D. Bratton, J. Biol. Chem. 269:22517-22523, 1994).

(ii) Relationship of PS egress to shedding of plasma membrane vesicles. Data from our laboratory helped establish that surface exposure of PS is intimately related to a process of Ca^{2+}-induced vesiculation of the plasma membrane, and that formation of such PS-rich plasma membrane "microparticles" contributes to expression of cellular procoagulant activity (C.-P. Chang, *et al.*, *supra*, 1993; P. J. Sims, *et al.*, *supra*,

1989; A. P. Bode, *et al.*, *supra*, 1985; R. F. A. Zwaal, *et al.*, *supra*, 1992; T. Wiedmer, *et al.*, *supra*, 1990; P. J. Sims, *et al.*, *J. Biol. Chem.* 263:18205-18212, 1988; K. K. Hamilton, *et al.*, *J. Biol. Chem.* 265:3809-3814, 1990; H. Sandberg, *et al.*, *Thromb. Res.* 39:63-79, 1985). This correlation between microparticle formation and surface exposure of PS suggested that the membrane fusion events generating these membrane vesicles underlie observed scrambling of plasma membrane PL, presumably through transient formation of H_{II}-phase PL (C. P. Chang, *et al.*, *supra*, 1993). Alternatively, prior egress of PS to the outer leaflet might create a mass imbalance that itself drives plasma membrane evagination and vesiculation (P.F. Devaux, *supra*, 1991). In this context, we and others have observed that PS migration to the cell surface can precede membrane vesiculation, and can occur without microparticle formation (G. E. Gilbert, *et al.*, *J. Biol. Chem* 266:17261-18269, 1991; F. Basse, *et al.*, *Biochemistry* 32:2337-2344, 1993; P. Gaffet, *et al.*, *supra*, 1995)). Our data suggested the participation of a calmodulin-dependent kinase in Ca^{2+}-induced vesiculation (T. Wiedmer, *et al.*, *Blood* 78:2880-2886, 1991). Involvement of a protein kinase in the cytoskeletal reorganization required for platelet microparticle formation has recently been confirmed (Y. Yano, *et al. Biochem. J.* 298:303-308, 1994). This suggests that whereas membrane fusion may contribute, it neither initiates nor is required for PL scramblase function.

(iii) Interaction of Ca^{2+} with anionic plasma membrane PL. Ion pairing of Ca^{2+} with the anionic PS headgroup might accelerate transbilayer migration by lowering the energy barrier to crossing through the hydrophobic membrane interior. However, Ca^{2+} does not directly induce transbilayer migration of PL in PS-containing membranes, except when mole% of PS is sufficient to induce an H_{II}-phase and vesicle-vesicle fusion (A.L. Bailey, et al., Biochemistry 33:12573-12580, 1994; B. de Kruijff, et al., Trends Biochem. Sci. 5:79-81, 1980). Thus ion pairing of Ca^{2+} with inner leaflet PS would not appear to directly induce PL flip/flop between membrane leaflets. Alternatively, Devaux and associates (J.-C. Sulpice, *et al.*,

J. Biol. Chem. 269:6347-6354, 1994; J.-C. Sulpice, *et al.*, *Biochemistry* 35:13345-13352, 1996) proposed that Ca^{2+} binding to phosphatidylinositol 4,5-bisphosphate (PIP2) induces transbilayer migration of other PL, based on the observation that adding PIP2 to RBC promoted Ca^{2+}-dependent transbilayer movement of PS. Nevertheless, subsequent studies revealed that this incorporation of exogenous PIP2 also induces enough membrane lysis to account for any apparent increase in transbilayer migration of PS (Bevers, *et al. Blood* 86:1983-1991, 1995).

(iv) Role of Ca^{2+}-sensitive protein(s) in PL redistribution. Elevation of $[Ca^{2+}]_c$ is known to give rise to marked changes in several cytoskeletal and membrane proteins that might affect the rate of movement of PL between plasma membrane leaflets. For example, several cytoskeletal proteins (including, spectrin and erythrocyte band 4.1)(A. M. Cohen, *et al.*, *Blood* 68:920-926, 1986; S. B. Sato, *et al.*, *Eur. J. Biochem.* 130:19-25, 1983; A. C. Rybicki, *et al.*, *J. Clin. Invest.* 81:255-260, 1988; K. A. Shiffer, *et al.*, *Biochim. Biophys. Acta* 937:269-280, 1988) have been shown to bind specifically to the PS headgroup, and this interaction potentially serves to trap PS in the inner leaflet of the plasma membrane (P. Comfurius, *et al.*, *Biochim. Biophys. Acta* 983:212-216, 1989). Breaking such interactions, as might occur through interaction of Ca^{2+} with the PS headgroup, or through proteolytic degradation of cytoskeletal proteins, would potentially dissociate PS from these endofacial contacts and thereby accelerate PS flop to the outer leaflet (P. F. Franck, *et al.*, *supra*, 1985). In this context, it has been reported that polyamines inhibit the endogenous PL scramblase activity of the RBC membrane, suggesting that these polycations inhibit interaction of Ca^{2+} at its endofacial membrane site (D. L. Bratton, *et al.*, *supra*, 1994; J.-C. Sulpice, et al. *supra*, 1996). Calpain-mediated proteolysis of components of the submembrane cytoskeleton can be temporally-correlated to membrane vesiculation and to surface exposure of PS. Nevertheless, inhibition of calpain does not prevent PS egress, and, Ca^{2+}-dependent PL scramblase activity is readily

demonstrated in resealed RBC ghosts and inside-out RBC membrane vesicles (IOV) washed free of calpains and other soluble proteases (P. Comfurius, et al., *supra*, 1990; T. Wiedmer, et al., *Biochemistry* 29:623-632, 1990; Fox, et al., *J. Biol. Chem.* 266:13289-13295, 1991; J. E. B. Fox, et al., *J. Cell Biol.* 111:483-493, 1990; P. F. J. Verhallen, et al., *Biochim. Biophys. Acta* 903:206-217, 1987; L. Dachary-Prigent, et al., *Blood* 81:2554-2565, 1993) On the other hand, the possibility that a membrane protein with inherent PL scramblase activity directly mediates Ca^{2+}-induced transbilayer migration of PL was suggested by the observation that this activity is inhibited by sulfhydryl oxidation of membrane proteins with PDA (P. Williamson, et al., *supra*, 1995). Consistent with this finding, we recently reported the purification and preliminary characterization of an integral RBC membrane protein that, when reconstituted in liposomes, mediates a Ca^{2+}-dependent transbilayer movement of PL mimicking plasma membrane PL reorganization evoked upon elevation of $[Ca^{2+}]_c$ (F. Bassé, *et al., J. Biol. Chem.* 271:17205-17210, 1996) Evidence that a protein of similar function must also be present in platelets was recently reported by Zwaal (P. Comfurius, *et al., Biochemistry* 35:7631-7634, 1996).

The Scott syndrome. Scott syndrome is a bleeding disorder described in a single patient that reflects impaired expression by activated platelets of the membrane sites that are required for normal assembly of the prothrombinase and tenase enzyme complexes (H. J. Weiss, *et al., Am. J. Med.* 67:206-213, 1979; H. J. Weiss, *Semin. Hematol.* 31:1-8, 1994). Platelets obtained from this patient secrete and aggregate normally when exposed to various agonists (ADP, thrombin, collagen, complement proteins C5b-9, or calcium ionophore), but when fully activated, exhibit a marked deficiency of membrane binding sites for factors Va and VIIIa, reflecting a concomitant reduction in the amount of surface-exposed PS (J. P. Miletich, *et al., supra*, 1979; J. Rosing, *et al., supra*,

1985; J. P. Sims, *et al.*, *supra*, 1989; S. S. Ahmad, *et al.*, *supra*, 1989).

Although Scott syndrome was originally described as an isolated platelet disorder, it is now clear that other blood cells from this patient, including erythrocytes and lymphocytes, are affected as well (E. M. Bevers, *et al. Blood* 79:380-388, 1992). Measurements that have been made by ourselves and others suggest that Scott platelets and erythrocytes contain normal amounts of PS and other phospholipids, and also exhibit normal aminophospholipid translocase activity (H. J. Weiss, *et al.*, 1979). These cells are defective, however, in their capacity to mobilize PS from inner to outer membrane leaflets in response to elevated $[Ca^{2+}]_i$, a response that is now thought to be required for normal prothrombinase and tenase assembly. A search for the molecular defect responsible for the Scott syndrome has to date failed to reveal abnormality in platelet or red cell proteins (probed by 2-dimensional electrophoresis); the agonist-induced elevation of $[Ca^{2+}]_i$ in Scott platelets is normal; and the calpain and transglutaminase activities of both Scott platelets and RBCs are indistinguishable from normal controls, as assessed by the Ca^{2+}-induced cleavage or cross-linking of cytoskeletal proteins (P. Comfurius, *et al.*, *Biochim. Biophys. Acta* 815:143-148, 1985).

We have established in *in vitro* culture EBV-transformed lymphoblast cell lines from this patient and have demonstrated that these immortalized cells exhibit the same phenotype of impaired Ca^{2+}-induced plasma membrane phospholipid scrambling that is characteristic of the platelets and erythrocytes in this disorder. Our data also establish that this defect propagates through multiple cell divisions and can be corrected by heterokaryon fusion with wild-type cells that exhibit normal plasma membrane phospholipid scrambling (H. Kojima, *et al. J. Clin. Invest.* 94:2237-2244, 1994).

Similar data have recently been reported for a second patient with Scott syndrome, and evidence for a familial inheritance of the cellular defect provided (F. Toti, *et al.*, *supra*, 1996)). This implies that the molecular basis for this

clinical disorder relates to a gene defect that results in impaired activity of a cellular protein that is expressed in a variety of cell lineages, and that this protein normally mediates the intracellular Ca^{2+}-dependent transbilayer movement (or "scrambling") of plasma membrane phospholipids that occurs in response to cell activation, apoptosis, or cell injury. We identify this protein as "phospholipid scramblase", "PL scramblase", and "P37". We mean for "P37" to be synonymous with "phospholipid scramblase or PL scramblase" and refer to these names interchangeably throughout the text.

The loss of normal PL scramblase function in patients with Scott syndrome may relate to either the abnormal expression of an inhibitor of the activity of PL scramblase, a deletion or loss of function mutation in the gene encoding P37 protein, or, a mutation affecting a cofactor of P37 that is required for normal expression of its PL scramblase activity.

Patients with the Scott syndrome defect display abnormal bleeding and a prolongation of the time required for blood clotting (H. J. Weiss, *Semin. Hematol.* 31:1-8, 1994). This implies that activation of PL scramblase is normally required for effective clot formation and for efficient hemostasis, whereas loss or inhibition of PL scramblase activity leads to retarded blood clotting. We therefore propose that the selective *activation* of PL scramblase function is of potential therapeutic value in the acceleration of hemostasis and in preventing blood loss, whereas the selective *inhibition* of PL scramblase function is of potential therapeutic value in certain thrombotic disorders characterized by excessive or inappropriate clot formation due to expression of plasma membrane procoagulant activity.

In this application, we identify the cellular component that functions to mediate the Ca^{2+}-dependent reorganization of plasma membrane phospholipids and describe methods for preventing egress of PS to the surface of activated, injured, or apoptotic cells.

BRIEF SUMMARY OF THE INVENTION

The present invention relates to the creation and use of antithrombotic and thrombostatic reagents that depend on the properties of a protein preparation that mediates Ca^{2+} dependent transbilayer movement of membrane phospholipids.

The present invention is a preparation of a protein, wherein the protein is a phospholipid scramblase and wherein the protein is approximately 35-37 kD as measured on a 12.5% SDS-polyacrylamide gel under reducing conditions. Preferably, the scramblase comprises residues 59 - 90 of SEQ ID NO:1. More preferably, the preparation comprises residues 1 - 188 of SEQ ID NO:1.

In another embodiment of the present invention, the PL scramblase comprises residues 59 - 90 of SEQ ID NO:1 with conservative or functionally equivalent substitutions.

In the most preferred embodiment of the present invention, the PL scramblase comprises residues 75-392 of SEQ ID NO:4 with conservative or functionally equivalent substitutions.

The present invention is also a DNA sequence encoding the PL scramblase. Preferably, this DNA sequence comprises the nucleic acids 223-1176 of SEQ ID NO:3.

The present invention is also an inhibitor of the PL scramblase activity of P37. This inhibitor may be an antisense nucleotide derived from the DNA sequence of P37. In another embodiment, the inhibitor is a peptide sequence that is a competitive inhibitor of P37 PL scramblase activity. Preferably, the inhibitor is a peptide that either binds to plasma membrane phosphatidylserine, prevents binding of Ca^{2+} to P37, or prevents its phosphorylation by cellular protein kinases. In another embodiment, the inhibitor is an antibody, preferably a monoclonal antibody, raised against P37.

The present invention is also a method for preventing the surface exposure of plasma membrane phosphatidylserine, phosphatidylethanolamine and cardiolipin on the surface of *in vitro* stored platelets or red blood cells. This method comprises the steps of adding an inhibitor of P37 PL

scramblase activity to the stored platelets or red blood cells.

The present invention is also a method for prolonging survival of transplanted organs comprising the step of adding an inhibitor of P37 PL scramblase activity to an organ perfusate during *in vitro* organ storage. The present invention is also a method for prolonging the survival of transplanted cells, tissues, and organs by genetically engineering the cells to be transplanted so as to alter their expression of plasma membrane P37 in order to reduce exposure of PS and other thrombogenic phospholipids at the plasma membrane surface, thereby reducing the risk of infarction due to fibrin clot formation.

The present invention is also a method for prolonging the *in vivo* survival of circulating blood cells (erythrocyte, platelets, lymphocyte, PMN's, and monocytes) comprising the step of preventing surface exposure of plasma membrane phosphatidylserine on the surface of the cells by exposing the blood cells to an inhibitor of P37 PL scramblase activity.

The present invention is also a method for preventing the procoagulant activities of erythrocytes in sickle cell disease comprising the step of inhibiting erythrocyte P37 in a sickle cell patient.

The present invention is also a method for treating autoimmune and inflammatory diseases comprising the step of treating a patient with an inhibitor of the PL scramblase activity of P37.

The present invention is also a method for diagnosing individuals with reduced or elevated capacity for platelet-promoted or erythrocyte-promoted fibrin clot activity comprising the step of quantitating the cellular expression of P37. This quantitation may take the form of immunoblotting using an antibody to P37, an ELISA assay using an antibody to P37, flow cytometric analysis of the binding of monoclonal antibody reactive against the predicted extracellular domain of P37 (residues 386 - 392 of sequence disclosed in SEQ ID NO:4) or using oligonucleotides derived from P37 cDNA and the polymerase chain reaction. In one method of the present

invention, the quantitation is performed by isolating P37 from a patient blood sample, measuring the amount of P37 isolated and comparing the measurement with a control sample. The measurement may be by isolating P37 from a patient blood sample and measuring via densitometry the amount of P37 protein electrophoresed in a stained electrophoretic gel.

It is an object of the present invention to provide a preparation of a PL scramblase.

It is another object of the present invention to provide an inhibitor of P37 PL scramblase activity.

It is another object of the present invention to provide an antithrombotic agent.

It is another object of the present invention to create cells, tissue, and organs for transplantation that have increased potential for survival and reduced potential for causing fibrin clot formation and vascular thrombosis when grafted into a recipient host.

Other objects, advantages and features of the present invention will become apparent after one of skill in the art reviews the specification, claims and drawing herein.

BRIEF DESCRIPTION OF THE SEVERAL VIEWS OF THE DRAWINGS

Fig. 1A is a bar graph depicting the amount of PL scramblase activity as a function of chromatography column fraction number. Fig. 1B represents a 12.5% SDS-PAGE in which the fractions described in Fig. 1A have been electrophoresed. The actual 37 kD bands are depicted by dark rectangles.

Fig. 2 is a diagram of the bidirectional movement of NBD-PS across lipid bilayers reconstituted with P37.

Fig. 3 is a graph describing the kinetics of PS translocation by reconstituted P37.

Fig. 4 is a graph describing calcium dependence of P37 activity in proteoliposomes and in erythrocyte IOVs.

DETAILED DESCRIPTION OF THE INVENTION

The present invention is the purification and characterization of an approximately 35-37 kD membrane protein from human erythrocytes that promotes a Ca^{2+}-dependent transbilayer redistribution of membrane phospholipids including PS and PC, with properties similar to the PL scramblase activity that is evoked upon elevation of Ca^{2+} in the cytosol of erythrocytes and other cells. We have named this membrane protein "P37." We mean for "P37" to be synonymous with "phospholipid scramblase or PL scramblase" and refer to these names interchangeably throughout the text. By "phospholipid scramblase or PL scramblase activity," we mean the Ca^{2+} dependent transbilayer movement of plasma membrane phospholipid.

In one embodiment, the present invention is a protein preparation of P37. Preferably, the protein comprises residues 59 - 90 of SEQ ID NO:1. More preferably, the PL scramblase comprises residues 1 - 188 of SEQ ID NO:1. Most preferably, the protein comprises residues 75 - 392 of SEQ ID NO:4.

In another embodiment, the protein comprises conservative substitutions or functionally equivalent residues of the residues described in the paragraph above. By "functionally equivalent" we mean that the equivalent residues do not inhibit or disrupt the activity of the PL scramblase preparation.

The examples below demonstrate one method of isolating P37 from human erythrocytes. After examination of the specification below, other methods of protein isolation will become apparent to one of skill in the art. The examples below also describe an assay for the measurement of PL scramblase activity. A suitable preparation of the present invention would have a PL scramblase activity of at least 10% that of the preparation described below in the examples. Preferably, the activity would be at least 50% that of the examples described below.

The present invention is also a recombinant DNA sequence encoding P37. A preferable DNA sequence encoding P37 would

comprise the nucleic acids of SEQ ID NO:2. A more preferable DNA sequence encoding P37 would comprise the nucleic acids 223 - 1176 of SEQ ID NO:3. One of skill in the art of molecular biology would know how to obtain DNA sequences encoding the PL scramblase. For example, one might sequence P37 directly via standard protein sequencing techniques as we report below in Example C. The peptide sequence could be analyzed to provide oligonucleotide probes for a human cDNA leukocyte library. (One such cDNA library is available from Invitrogen in a pCDNA3 vector.)

Residues 59 - 90 of SEQ ID NO:1 are the amino acid sequence that we obtained of a cyanogen bromide fragment of the PL scramblase. SEQ ID NO:1 is the translated product of ATCC clone 962235 and is a longer amino acid sequence containing residues 59 - 90. Residues 75 - 392 of SEQ ID NO:4 is the predicted full-length sequence of the P37 polypeptide, based on analysis of the open reading frame of SEQ ID NO:3.

By use of probes obtained from other domains of the PL scramblase one would then be able to isolate a cDNA clone encoding the entire P37 protein sequence. Sequence identified as SEQ ID NO:3 contains the entire open reading frame encoding P37 as well as flanking residues of 5' and 3' untranslated sequence. The full-length translation of SEQ ID NO:3 is identified as SEQ ID NO:4. (The symbol Xaa indicates a stop.) Analysis of the open reading frame of this sequence identifies residues 75 - 392 of SEQ ID NO:4 as comprising the full-length protein sequence of P37. No signal peptide sequence is identified between residues 75 -392 of SEQ ID NO:4, indicating that this is also likely to be the length of the mature polypeptide after post-translational processing. The calculated molecular weight of the polypeptide containing residues 75-392 of SEQ ID NO:4 is 35,049 daltons and closely approximates the molecular weight of P37 of 35-37 kDa as estimated by apparent mobility in SDS-PAGE.

Most preferably, the preparation would have at least 90% the activity of the PL scramblase preparation described below.

In one embodiment of the invention, the PL scramblase is isolated from erythrocyte membranes. In another embodiment,

the protein is produced by bacteria cells, such as *E. coli* cells, insect cells, or yeast, preferably *in vitro* cultures that are transfected with plasmid or viral vectors containing cDNA sequences identified at SEQ ID NO:2 in the correct reading frame (3) or cDNA sequences identified at SEQ ID NO:3 in correct reading frame (1). The vector can be chosen from among protein expression vectors known to those skilled in the art. Preferable viral vectors include retrovirus, adenovirus, and baculovirus vectors.

The present invention is also an inhibitor of the PL scramblase activity of P37. In one embodiment, the inhibitor is an antisense nucleotide derived from the DNA sequence encoding P37. One of skill in the art would know how to create such an antisense nucleotide from the cDNA sequence of P37. In another embodiment, the inhibitor is an antibody, preferably a monoclonal antibody, raised against P37. One of skill in the art would know how to make an antibody preparation from the purified protein preparation described below. For example, one could isolate the purified P37 protein from the electrophoretic gel band and use this preparation to inject into an antibody-producing host, such as a rabbit, sheep, goat, rat, or mouse. In addition to polyclonal immune serum, the antibody-producing splenic lymphocytes from such animals can be obtained to prepare monoclonal antibody reactive with specific peptide segments of P37. Such methods are well-known to those skilled in the art and include myeloma fusion and the *in vitro* culture, cloning and selection of the resulting hybridoma cells to obtain monoclonal IgG. In a preferred embodiment, recombinant antibody fragments consisting of antigen-binding Fv or ligated single chain Ig ScFv can be prepared from a cDNA library derived from the polyA mRNA of the immune splenic lymphocytes. In this embodiment, PCR is used to selectively amplify cDNA encoding the CDR3 hypervariable domains of the heavy and light Ig chains, this cDNA is then ligated into appropriate phage expression vectors, and the recombined or fused single chain Fv are then expressed as soluble protein and on the surface of the phage particles that are propagated in E. Coli or other

suitable host cell. The resulting express recombined monoclonal Ig Fv on their surface and can be panned and cloned to isolate unique cDNA encoding for specific monoclonal reagents that bind to select epitopes on P37 antigen. This unique cDNA can then amplified and used to express large quantities of the desired monoclonal antibody reagent in an appropriate expression system. In another embodiment, the anti-P37 Fv reagent is humanized to reduce its antigenicity for purposes of injection into a human host. The humanized Fv is prepared by converting the nucleotide segments of the murine-derived cDNA to the homologous human sequence in those segments of the heavy and light chain Ig polypeptides that are flanking on either side of the actual antigen combining site. The methods for preparation of these various recombinant immunoglobulin reagents are well-known to those skilled in the art and are also commercially available in kit form (e.g. Pharamacia ScFv).

In another embodiment, the inhibitor is a peptide derived from P37. This peptide sequence is an antagonist or competitive inhibitor of P37 PL scramblase activity. The inhibitor activity could take the form of competing with the binding of native P37 with plasma membrane phosphatidylserine or competing with native P37 in calcium binding. One of skill in the art would realize that one could examine different peptide fragments of P37 for suitability as a competitive inhibitor.

The present invention is also a method for preventing the surface exposure of plasma membrane phospholipids, such as phosphatidylserine, phosphatidylethanolamine and cardiolipin, on the surface of *in vitro* stored platelets or red blood cells by adding an inhibitor of the PL scramblase activity of P37 to the stored platelets or red blood cells.

The present invention is also a method for prolonging survival of transplanted organs and grafts comprising the step of adding an inhibitor of P37 PL scramblase activity to an organ perfusate during *in vitro* organ storage. The present invention is also a method for prolonging the survival of transplanted cells, tissues, and organs by genetically

engineering the cells to be transplanted so as to alter their expression of plasma membrane P37 in order to reduce exposure of PS and other thrombogenic phospholipids at the plasma membrane surface, thereby reducing the risk of infarction due to fibrin clot formation.

Therefore, in one embodiment, the present invention is a genetically engineered cell for transplantation into a human or animal wherein the cell has a lowered PL scramblase expression. Preferably, the cell expresses no PL scramblase. Preferably, this cell comprises a nucleotide molecule which is expressed by the cell and which codes for protein inhibiting the activity of PL scramblase. In another preferable embodiment, the promotor of the PL scramblase gene is altered to either increase or decrease the expression of the gene. One of skill in the art of molecular biology would envision methods to create these altered cells.

Preferably, the engineered cell is selected from the group consisting of endothelial cells, fibroblasts, epithelial cells, skeletal cells, cardiac and smooth muscle cells, hepatocytes, pancreatic islet cells, bone marrow cells, astrocytes, and Schwann cells. The present invention is also a prosthesis for implantation in an animal or human having the genetically engineered cells attached thereto. In one embodiment, the prosthesis is a vascular graft.

The present invention is also a method for prolonging the *in vivo* survival of circulating blood cells comprising the step of preventing surface exposure of plasma membrane phosphatidylserine on the circulating blood cells plasma membrane P37. One may also wish to prevent the procoagulant properties of erythrocytes in sickle cell disease by inhibiting erythrocyte P37 in a sickle cell patient.

The present invention is also a method for treating autoimmune and inflammatory diseases, such as disseminated intravascular coagulation, vascular thrombosis, fibrin generation during cardiopulmonary bypass procedures, rheumatoid arthritis, systemic lupus erythematosus, thrombotic thrombocytopenic purpura, heparin-associated thrombosis, and

organ transplant rejection comprising the step of treating a patient with an inhibitor of the PL scramblase activity P37.

The present invention is also a method for diagnosing individuals with reduced or elevated capacity for platelet-promoted or erythrocyte-promoted fibrin clot activity by quantitating the level of cellular expression of P37 in the individual. This method may be performed by using an antibody to P37 in an immunoblot or ELISA method. The method may also be performed using oligonucleotides derived by P37 cDNA in the polymerase chain reaction. In another embodiment, the method may be performed by isolating P37 from a whole blood sample, measuring the amount of P37 isolated and comparing the measurement with a control sample.

One may wish to use the protein preparation of the present invention as a hemostatic agent by topically applying the protein preparation to a wound area in a freely bleeding patient.

EXAMPLES

A. Experimental Procedures.

Abbreviations used. **PL**, phospholipid(s); **PC**, phosphatidylcholine; **PS**, phosphatidylserine; **PE**, phosphatidylethanolamine; **NBD-PC**, 1-oleoyl-2-[6(7-nitrobenz-2-oxa-1,3-diazol-4-yl)amino]caproyl-*sn*-glycero-3-phosphocholine; **NBD-PS**, 1-oleoyl-2-[6(7-nitrobenz-2-oxa-1,3-diazol-4-yl)amino]caproyl-*sn*-glycero-3-phosphoserine; **OG**, N-Octyl-β-D-glucopyranoside; **PIP_2**, phosphatidylinositol 4,5-bisphosphate; **APLTase**, aminophospholipid translocase; **IOVs**, inside-out vesicles.

Materials. Egg yolk phosphatidylcholine (PC), brain phosphatidylserine (PS), 1-palmitoyl 2-oleoyl phosphatidic acid, 1-oleoyl-2-[6(7-nitrobenz-2-oxa-1,3-diazol-4-yl)amino]caproyl-*sn*-glycero-3-phosphocholine (NBD-PC) and 1-oleoyl-2-[6(7-nitrobenz-2-oxa-1,3-diazol-4-yl)amino]caproyl-*sn*-glycero-3-phosphoserine (NBD-PS) were obtained from Avanti Polar Lipids. N-Octyl-β-D-glucopyranoside (OG) was purchased

from Calbiochem. Sodium dithionite ($Na_2S_2O_4$, Sigma) was freshly dissolved in 1 M Tris pH 10 at a concentration of 1 M.

PL Scramblase isolation. Human erythrocytes were obtained from The Blood Center of Southeastern Wisconsin. Washed erythrocytes were lysed in 10 vol of ice cold 5 mM sodium phosphate, 1 mM EDTA, pH 7.4, washed free of hemoglobin, and solubilized at a final protein concentration of 3.5 mg/ml in the presence of 100 mM OG in buffer A (20 mM Tris, 0.1 mM EGTA, pH 7.4). After incubation for 30 minutes at 4°C with continuous stirring, samples were centrifuged at 100,000 x g for 30 minutes. The cytoskeleton-depleted supernatants were loaded on a Poros 20 HQ anion-exchange column (PerSeptive Biosystems) equilibrated with buffer B (20 mM Tris, 0.1 mM EGTA, 50 mM OG, pH 7.4). Elution was performed with a gradient of 0-400 mM NaCl in buffer B. Active fractions were pooled, subjected to a buffer exchange by PD 10 gel filtration (Pharmacia) into buffer C (50 mM acetate, 0.1 mM EGTA, 50 mM OG, pH 5.0), and loaded on a Poros 20 HS cation-exchange column (PerSeptive Biosystems) equilibrated in buffer C. The breakthrough fraction was passed through a PD 10 column equilibrated in buffer D (25 mM PIPES, 1 mM $CaCl_2$, 50 mM OG, pH 6.0) and loaded on a Reactive Red 120 6% agarose column (Sigma). Elution was performed with a gradient of 0-1.5 M NaCl in buffer D. Active fractions were pooled and further purified on a Bio-Sil SEC-250 size exclusion column (Bio-Rad) equilibrated with buffer D in which $CaCl_2$ was replaced by 0.1 mM EGTA. Due to losses encountered through size exclusion chromatography, enriched fractions of PL scramblase were routinely prepared by replacing this step by a second anion exchange fractionation on Mono Q (Pharmacia). All column chromatography procedures were performed at room temperature on a BioCAD 20 perfusion chromatography workstation (PerSeptive Biosystems).

Reconstitution into proteoliposomes. A mixture of PC and PS (9:1 molar ratio) was dried under a stream of nitrogen and resuspended in buffer E (100 mM Tris, 100 mM KCl, 0.1 mM EGTA,

pH 7.4) by vigorous vortexing. Protein samples were mixed with liposomes at a final lipid concentration of 4 mg/ml in the presence of 60 mM OG. Detergent was removed by dialysis overnight at 4°C against 200 vol of buffer E containing 1 g/L SM2-Biobeads (BioRad).

Labeling of proteoliposomes with NBD-PL. Proteoliposomes were labeled with fluorescent NBD-PC or NBD-PS (0.25 mole%) selectively incorporated into outer, inner, or both membrane leaflets (E. M. Bevers, *et al.*, *supra*, 1995; J. C. McIntyre and R. G. Sleight, *Biochemistry* 30:11819-11827, 1991). Outer leaflet-labeled proteoliposomes were prepared by addition of NBD-PL (in DMSO) to the pre-formed proteoliposome suspension. Symmetrically-labeled proteoliposomes were prepared by adding NBD-PL to the PC-PS mixture in $CHCl_3$, before drying lipids under nitrogen (*above*). Addition of protein sample in OG and dialysis against buffer E was then performed as above. Proteoliposomes with fluorescent NBD-PL located exclusively in the inner leaflet of the bilayer were obtained by incubating symmetrically-labeled proteoliposomes with 20 mM dithionite for 1 minute, in order to chemically and irreversibly quench fluorescent analogs located in the outer leaflet (J. C. McIntyre and R. G. Sleight, *Biochemistry* 30:11819-11827, 1991). Vesicles were gel filtered in buffer E to remove unincorporated material and used immediately for assay of PL scramblase activity.

PL Scramblase activity. PL scramblase activity was measured using a modification of an assay previously described (E. M. Bevers, *et al.*, *supra*, 1995; J. C. McIntyre and R. G. Sleight, *supra*, 1991). Routinely, NBD-PL outside-labeled proteoliposomes (0.4 mg/ml PL final concentration) were incubated at 37°C in buffer E in presence or absence of 2 mM $CaCl_2$. At times indicated in figure legends, proteoliposomes were diluted 25-fold in buffer E containing 4 mM EGTA, and transferred to a stirred fluorescence cuvet at 23°C. Initial fluorescence was recorded (SLM Aminco 8000 spectrofluorimeter; excitation at 470 nm, emission at 532 nm), and 20 mM

dithionite added with fluorescence continuously monitored for total of 90 seconds. The difference in residual (non-quenchable) fluorescence observed for samples preincubated at 37°C in presence vs. absence of 2 mM $CaCl_2$ was attributed to Ca^{2+}-induced change in NBD-PL located in the outer leaflet, and hence accessible to dithionite (J. C. McIntyre and R. G. Sleight, *supra*, 1991). Maximum (100%) dithionite quenching was determined by addition of 1% Triton X-100. In all cases, NBD fluorescence was corrected for photobleaching (<6%; measured in absence of dithionite). Ionized [Ca^{2+}] (Fig. 4) was calculated using FreeCal version 4.0 software (generously provided by Dr. Lawrence F. Brass, University of Pennsylvania, Philadelphia, PA).

Trypsination. Samples containing active PL scramblase were incubated for 3 hours at 37°C in the presence of 2 μg/ml trypsin in 100 mM Tris, 0.1 mM EGTA, pH 8.4, and the reaction was stopped by addition of 1 mM diisopropylfluorophosphate. Samples incubated in absence of trypsin or in presence of trypsin plus 4 μg/ml soybean trypsin inhibitor served as controls. Residual PL scramblase activity of each sample was then determined following reconstitution into NBD-PS outside-labeled liposomes. Residual PL scramblase detected in trypsin-treated samples was normalized to that observed for identically matched samples incubated without trypsin.

Preparation of erythrocyte IOVs. Erythrocyte inside out vesicles (IOVs) were prepared essentially by methods described by Steck and Kant (T. L. Steck and J. A. Kant, *Methods Enzymol*. 31a:172-180, 1974) with minor modifications. Assays for acetylcholinesterase (outside) and glyceraldehyde 3-phosphate dehydrogenase accessibility (inside) (T. L. Steck and J. A. Kant, *supra*, 1974) confirmed that >90% of these IOVs were properly oriented and resealed.

PL Scramblase activity in IOVs. IOVs were suspended in 10 mM Tris, 140 mM KCl, 7.5 mM NaCl, 0.1 mM EGTA, pH 7.4 at 100 μg protein/ml. NBD-PLs (PC or PS) were added to a final

0.25 mole% of total PL. After incorporation of label, IOVs were incubated in presence 0-2 mM $CaCl_2$ for 15 minutes at 37°C. For each sample, percentage of NBD-PL displaced from the external to inward-facing membrane leaflet was determined by the BSA "back-exchange" method as previously described (E. M. Bevers, *et al.*, *supra*, 1995). PL scramblase activity was evaluated by comparing the amount of NBD-PL moved to the inward-facing leaflet of IOVs in the presence of Ca^{2+} to that observed in the presence of 0.1 mM EGTA. Transport of dithionite by erythrocyte membrane anion exchanger precludes use of this quencher to monitor NBD-PL distribution in IOVs (T. Pomorski, *et al.*, *Mol. Membr. Biol.* 11:39-44, 1994).

B. Results and Discussion

Purification and membrane reconstitution of erythrocyte PL scramblase. Human erythrocyte membrane proteins depleted of cytoskeleton were solubilized with OG and initially fractionated by anion exchange chromatography. Each eluting fraction was reconstituted into PL vesicles and then 0.25 mole% NBD-PS added to the outer leaflet. After incubation in the presence of either 0 or 2 mM Ca^{2+}, the time-dependent distribution of NBD-PS between inner and outer leaflets was determined (see *Experimental Procedures* and below). A Ca^{2+}-dependent movement of NBD-PS from outer to inner membrane leaflets was detected in only those vesicles reconstituted with proteins eluting from Poros 20 HQ between 125 mM-200 mM NaCl. Such activity was not detected in any other column fraction, including those enriched with the predominant erythrocyte membrane band 3 protein, nor was this activity found in unbound flow-through fractions (not shown). Of note, erythrocyte band 3 protein has been reported to exhibit intrinsic "flipping activity" for anionic PL, based on the capacity of the anion transport inhibitor 4,4'-diisothiocyanostilbene-2,2'-disulfonic acid to further attenuate the slow spontaneous (Ca^{2+}-*independent*) migration PL across the erythrocyte membrane (A. Vondenhof, *et al.*, *Biochemistry* 33:4517-4520, 1994).

Through subsequent purification, this PL scramblase activity was found to co-purify with a ~37 kDa protein band that we were able to enrich to apparent homogeneity (Fig. 1). Fig. 1 illustrates final stage of purification of erythrocyte PL scramblase activity. Pool of activity eluting from Reactive Red 120 was concentrated and applied to a 600 x 7.5 mm BioSil SEC250 column. Flow rate was 0.2 ml/min and 1.0 ml fractions were collected. 100 µl aliquots of each column fraction were reconstituted into liposomes containing NBD-PS exclusively in the outer leaflet and assayed for PL scramblase activity. Samples were also concentrated 10-fold and analyzed by 12.5% SDS-PAGE under reducing conditions, developed with silver staining. Relative PL scramblase activity is plotted directly above the corresponding gel lane. Only data for fractions eluting about peak of activity (fraction #12) are shown. See Experimental Procedures. Fractions 11 and 13 contained additional bands not represented in Fig. 1B.

The activity of this PL scramblase was destroyed by trypsin (Table 1), confirming that activity detected in the reconstituted proteoliposome derived from the purified protein fraction, and not other potentially co-eluting material. In this context, PL scramblase activity has recently been attributed to an interaction of Ca^{2+} directly with membrane lipid. As noted above (*Introduction*), Sulpice, *et al.* (J. C. Sulpice, *et al.*, *supra*, 1994) concluded that Ca^{2+}-binding to PIP_2 located in the inner leaflet of the erythrocyte membrane induces transbilayer PL scrambling, although this conclusion was not supported by a subsequent study (E. M. Bevers, *et al.*, *supra*, 1995). Alternatively, Bratton proposed that cytoplasmic polyamines contribute to maintenance of PL asymmetry by shielding anionic head groups from Ca^{2+}, and that enhanced transbilayer movement of PL might arise through loss of polyamine-membrane associations at increased intracellular Ca^{2+} (D. L. Bratton, *supra*, 1994). Although our studies do not address a potential influence of these cytoplasmic components on PL scramblase activity, it should be noted that polyamines would be removed through our lysis and solubilization procedures. Finally, sensitivity of PL

scramblase to inactivation by trypsin (Table 1) excludes the possibility that the activity we observe arises from contaminating PIP_2 or polyamine. Although data of Fig. 1 and Table 1 suggest that PL scramblase is a low abundance integral membrane polypeptide of ~37 kDa, we cannot now exclude the possibility that observed activity resides in a trace co-purifying membrane protein that goes undetected by silver staining.

Characteristics of PL transport mediated by isolated PL scramblase. Proteoliposomes reconstituted with purified PL scramblase remained impermeant to dithionite and stable to spontaneous NBD-PS exchange in absence of Ca^{2+} (Fig. 2).

Fig. 2 illustrates bidirectional movement of NBD-PS across lipid bilayers reconstituted with PL scramblase. PL scramblase was reconstituted into 90% PC, 10% PS membranes trace-labeled with 0.25% NBD-PS, initially located either in the inner membrane leaflet (*inside-labeled*), randomly distributed between leaflets (*symmetrically-labeled*), or exclusively in the outer leaflet (*outside-labeled*). Vesicles were incubated 3 hours at 37°C in presence of either 0.1 mM EGTA (*dotted lines*) or 2 mM Ca^{2+} (*solid lines*) and reaction stopped with EGTA. Distribution of NBD-PS in outer leaflet was determined by quenching with 20 mM dithionite, time of addition indicated by arrow (see Experimental Procedures). *Ordinate* denotes NBD fluorescence, normalized to t=0; *abscissa* denotes fluorescence acquisition time (sec). *Dashed line* indicates level of fluorescence detected after addition of 1% Triton X-100 to each vesicle suspension in presence of dithionite (100% quenching). In this experiment, observed Ca^{2+}-dependent transbilayer movement of NBD-PS equaled 8±2% (*inside labeled*), 0±1% (*symmetrically-labeled*), and 8±1% (*outside-labeled*). Fig. 2 illustrates the data of single experiment, representative of at least three separate experiments so performed.

As also illustrated by Fig. 2, Ca^{2+}-induced PL scramblase activity was observed to be bidirectional, induced sequestration of NBD-PS initially distributed only in the

outer membrane leaflet equaling induced exposure of NBD-PS initially distributed only in the inner leaflet. In proteoliposomes prepared with NBD-PS randomly distributed between membrane leaflets, no movement of probe was detected upon Ca^{2+} addition, consistent with one-for-one PL exchange between membrane leaflets. Similar results were obtained when NBD-PC substituted for NBD-PS, and movement of either NBD-labeled PL was unaffected by replacement of matrix PS by phosphatidic acid (data not shown). These results imply that the PL scramblase activity exhibited by lipid vesicles reconstituted with this 37 kDa protein is non-selective for PS versus PC, and facilitates both inward and outward movement of PL between membrane leaflets. Assuming that movement of PL initiated by addition of Ca^{2+} arises through activation of only those proteins oriented to expose Ca^{2+}-binding site(s) at the external face of the liposome, these data imply that each PL scramblase mediates bidirectional and non-selective one-for-one exchange of PL between membrane leaflets. Although it has been suggested that elevation of cytosolic Ca^{2+} initiates a vectorial movement of PS and PE from inner to outer plasma membrane leaflets (Bassé, *et al.*, *supra*, 1993; P. Gaffet, *et al.*, *supra*, 1995), more recent data suggest that this apparent egress of inner leaflet PS arises through random bidirectional exchange of PL residing in both leaflets of the plasma membrane (P. Williamson, *et al.*, *supra*, 1992; E. F. Smeets, *et al.*, *supra*, 1994; P. Williamson, *et al.*, *supra*, 1995), consistent with the activity we observe in PL scramblase reconstituted proteoliposomes. The fact that these proteoliposomes remain impermeant to dithionite (see Fig. 2), excludes the possibility that this apparently random and bidirectional movement of lipid arises through lytic disruption of lipid bilayer. Nevertheless, we cannot exclude the possibility that an initially vectorial transport of PL in these small vesicles is masked by a counterbalancing exchange to maintain mass balance between membrane leaflets, as might be imposed by head group packing constraints.

The mechanism by which Ca^{2+} interaction with this protein initiates transbilayer movement of PL remains unresolved. To

be noted, we observed only a requirement for Ca^{2+} with no requirement for either Mg^{2+} or ATP. In presence of 2 mM Ca^{2+}, lipid movement catalyzed by PL scramblase obeyed pseudo-first order kinetics with initial rates that increased with concentration of protein, consistent with a facilitated transport or carrier mechanism (Fig. 3).

Fig. 3 diagrams the kinetics of PS translocation by reconstituted PL scramblase. Proteoliposomes were prepared with a constant amount of PL and 2-fold dilutions of purified PL scramblase (*Experimental Procedures*). NBD-PS (0.25 mole%) was added to outer leaflet and labeled proteoliposomes incubated at 37°C in presence of 2 mM Ca^{2+}. At times indicated (*abscissa*) samples were analyzed for the amount of NBD-PS that migrated from outer to inner leaflets, according to assay shown in Fig. 2. All data are corrected for spontaneous transbilayer migration of NBD-PS (≤1% hr^{-1}) measured for identical samples incubated with 0.1 mM EGTA, substituting for Ca^{2+}. Fig. 3 illustrates the data at highest protein concentration (○); 2-fold (■); 4-fold (●) and 8-fold dilutions (□) are indicated. Experimental points at each protein concentration were fitted to first-order exponential rate equation (Sigma Plot; *dotted lines*). *Insert:* Derived initial rates (V_i; *ordinate*) plotted against normalized protein concentration (*abscissa*). Data of single experiment, representative of two so performed.

Derived rate constants for transbilayer lipid movement (at 37°C) ranged between 0.45 - 0.74 hr^{-1} over an eight-fold range of protein/lipid ratio. Whereas this activity deviated from anticipated linearity with increased added protein per lipid (insert, Fig. 3), this may reflect an inherent limitation in efficiency of protein reconstitution into the liposome membrane (*below*). The apparent rate of Ca^{2+}-evoked transbilayer movement attained in the reconstituted system ($t_{½}$ ~2 hr; Fig. 3) is approximately one-fourth of the observed rate of PL scrambling ($t_{½}$ ~0.5 hr; P. Williamson, *et al.*, *supra*, 1992) induced in Ca^{2+}-ionophore treated erythrocytes, suggesting somewhat reduced activity in the reconstituted system. Whether this reduced activity reflects partial

denaturation of PL scramblase, or, our inability to incorporate amounts of this protein equivalent to that present in the erythrocyte membrane, remains to be determined.

Calcium dependence. In order to confirm that the activity of reconstituted PL scramblase mimicked intrinsic PL scramblase activity of the erythrocyte membrane, we compared $[Ca^{2+}]$-dependence of PL movement in proteoliposomes reconstituted with purified protein to that observed in erythrocyte IOVs (Fig. 4). Fig. 4 diagrams Ca^{2+} dependence of PL scramblase activity in proteoliposomes & erythrocyte IOVs. PL scramblase activity of reconstituted proteoliposomes (*closed symbols*) and erythrocyte IOVs (*open symbols*) was determined as described in *Experimental Procedures*, and plotted as function of external free $[Ca^{2+}]$ (●,○). Data for each sample were normalized to maximum PL scramblase activity, measured at 2 mM Ca^{2+}. *Squares* (■,□) denote PL scramblase activity detected when Mg^{2+} substituted for Ca^{2+}. Data of single experiment, representative of three so performed.

In both IOVs and isolated protein reconstituted liposomes, PL scramblase activity was found to obey a sigmoidal dose-response to Ca^{2+}, with saturation at ~100 μM $[Ca^{2+}]$, and half-maximal activities attained between 20-60 μM Ca^{2+}. In both cases, little effect of Mg^{2+} was observed. These results are similar to data previously reported for Ca^{2+}-induced movement of plasma membrane PL in ionophore-treated erythrocytes, resealed erythrocyte ghosts, and other cells (H. Kojima, *et al.*, *Biochemistry* 33:4517-4520, 1994; B. Verhoven, *et al.*, *Biochim. Biophys. Acta* 1104:15-23, 1992).

PL scramblase activity found in erythrocyte membranes is also observed in platelets, lymphocytes and other cells and is thought to play a central role in initiation of fibrin clot formation and in recognition of apoptotic and injured cells by the reticuloendothelial system. It is not known whether the same protein is responsible for PL scramblase activity found in other cells, or whether such proteins are cell-specific. A protein common to all blood cells is suggested by the

observation that the genetic defect in PL scramblase activity arising in Scott syndrome affects all hematologic lineages. Further insight into the cellular distribution of the 37 kDa protein that we have now isolated from normal erythrocytes awaits purification of sufficient protein for sequencing, and to develop specific antibodies.

Table 1. Inactivation of PL scramblase by trypsin

Treatment		PL scramblase activity[a] (% of control)
Trypsin	Soybean Trypsin Inhibitor	
-	-	(100)
-	+	100
+	-	13
+	+	100

[a]PL scramblase in response to 2 mM Ca^{2+}, expressed as percent of activity of untreated control. Incubation with trypsin and reconstitution of digested protein into liposomes for PL scramblase assay are detailed under *Experimental Procedures*. Range of measured activities ± 5%. Representative of two separate experiments.

C. Amino Acid and Nucleotide Sequence of the PL Scramblase. We purified the PL scramblase by the methods described above. The resulting protein was subjected to further purification by sodium dodecyl sulfate polyacrylamide gel electrophoresis, the protein band visualized by staining with Coomassie brilliant blue, and a slice of the wet gel containing the P37 protein band migrating between ~35-37 kDa excised. This gel slice was sent to the Protein and Carbohydrate Structure Facility at University of Michigan, Ann Arbor, Michigan for cyanogen bromide digestion and internal peptide sequencing. Peptide sequence was obtained from a 2 kD cyanogen bromide fragment derived from the protein contained in the gel slice. The amino acid sequence we obtained is disclosed in residues 59 - 90 of SEQ ID NO:1.

We obtained an exact match for these residues with a translation product of a cDNA reported in Genbank at AA14325.

This sequence includes the predicted methionine (residue 58 of SEQ ID NO:1) located 5' to the preferred locus of cyanogen bromide peptide bond cleavage, yielding a total of 33 residues of continuous identity between the deduced partial internal sequence from P37 and the translation product of the cDNA clone reported in Genbank at AA143025. The entire nucleotide sequence of this cDNA clone (ATCC 962235) is reported at SEQ ID NO:2. Sequence reported at SEQ ID NO:1 is the entire translated sequence of SEQ ID NO:2, using the correct translation reading frame that we deduced from comparison of the cDNA clone with residues 58 - 90 of SEQ ID NO:1.

Using methods standard to those skilled in the art, we probed human leukocyte, B-lymphocyte, fetal adrenal, and K562 (a human erythro-leukemic cell line) cDNA libraries by PCR, using oligonucleotide primers designed to anneal specifically to SEQ ID NO:2. The PCR reactions revealed that cDNA overlapping SEQ ID NO:2 of the expected number of residues was contained in each of these cDNA libraries. This result indicates that mRNA encoding sequence contained in P37 protein purified from human erythrocyte membrane is also expressed in multiple human cell lines of both erythroid and non-erythroid origin. Based on evidence for the relative abundance of cDNA overlapping SEQ ID NO:2 in the Clontech lambda gt11 K562 library, we selected this library to amplify additional cDNA sequence flanking SEQ ID NO:2.

Using methods standard to those skilled in the art, we PCR amplified the flanking DNA sequences 5' and 3' to sequence ID NO:2 from the K562 lambda gt11 cDNA library (Clontech, Inc) with oligonucleotide primers designed to anneal to sequence ID NO:2 in combination with oligonucleotide primers designed to anneal to regions of the Clontech lambda gt11 vector in the vicinity of the multiple cloning site of the vector. The resulting PCR-amplified cDNA was purified and sequenced. From these PCR-amplified 5' and 3' flanking sequences, partial overlapping identities were found in cDNA reported in Genbank at AA143025, AA056199, AA054476, AA151006, AA171663, D61890, H42131, R76171, R66275, R82537, W90481 and these multiple sequences used in combination with direct sequence obtained

from our PCR products to deduce and to confirm the sequence reported at SEQ ID NO:3.

SEQ ID NO:4 is the entire translated sequence of SEQ ID NO:3, using the correct translation reading frame that we deduced from comparison of the cDNA clone with peptide residues 58 - 90 of SEQ ID NO:1.

The open reading frame of the sequence reported at SEQ ID NO:3 predicts a polypeptide of 318 amino acids (identified as residues **Met75- Trp392** of SEQ ID NO:4) with the calculated molecular weight of 35,049 and theoretical isolectric point (pI) = 4.83. This closely approximates the experimentally estimated molecular weight and isolectric point of P37 PL scramblase purified from erythrocyte membranes. Thus we identify residues 75 - 392 of SEQ ID NO:4 as P37, human plasma membrane PL scramblase and deduce that this same protein is expressed in both erythroid and non-erythroid human cells.

Analysis of the sequence contained in P37 (identified as residues 75 - 392 of SEQ ID NO:4) predicts a membrane protein with a cytoplasmic N-terminus and a single transmembrane helix formed by residues 365 - 383, with residues 384 - 392 projecting from the cell surface and residues 75 - 364 internal to the cell. A site for phosphorylation by protein kinase C or other serine/threonine protein kinase is predicted at P37 Thr residue 235 (all residue numbers for P37 given according to sequence identified at SEQ ID NO:4). It has been observed that activity of PL scramblase is reduced in cells depleted of ATP (Martin, *et al.*, *J. Biol. Chem.* 270:10468-10474, 1995), and that inhibitors of Ca^{2+}/calmodulin kinases reduce platelet plasma membrane microparticle formation (Wiedmer, et al., Blood 78:2880-2886, 1991), which is consistent with the regulation of the activity of PL scramblase by a phosphorylation of P37 mediated by one or more intracellular protein kinases.

SEQUENCE LISTING

(1) GENERAL INFORMATION:

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(G) TELEPHONE: (414) 456-4402
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(ii) TITLE OF INVENTION: A PROTEIN PREPARATION THAT MEDIATES Ca^{2+}-DEPENDENT TRANSBILAYER MOVEMENT OF PLASMA MEMBRANE PHOSPHOLIPID AND INHIBITORS THEREOF

(iii) NUMBER OF SEQUENCES: 4

(iv) CORRESPONDENCE ADDRESS:
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(v) COMPUTER READABLE FORM:
(A) MEDIUM TYPE: Floppy disk
(B) COMPUTER: IBM PC compatible
(C) OPERATING SYSTEM: PC-DOS/MS-DOS
(D) SOFTWARE: PatentIn Release #1.0, Version #1.25

(vi) CURRENT APPLICATION DATA:
(A) APPLICATION NUMBER:
(B) FILING DATE:
(C) CLASSIFICATION:

(viii) ATTORNEY/AGENT INFORMATION:
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(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 188 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

```
Ala Phe Gln Gly Pro Pro Gly Tyr Ser Gly Tyr Pro Gly Pro Gln Val
1               5                  10                  15

Ser Tyr Pro Pro Pro Pro Ala Gly His Ser Gly Pro Gly Pro Ala Gly
            20                  25                  30

Phe Pro Val Pro Asn Gln Pro Val Tyr Asn Gln Pro Val Tyr Asn Gln
        35                  40                  45
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Pro Val Gly Ala Ala Gly Val Pro Trp Met Pro Ala Pro Gln Pro Pro
50 55 60

Leu Asn Cys Pro Pro Gly Leu Glu Tyr Leu Ser Gln Ile Asp Gln Ile
65 70 75 80

Leu Ile His Gln Gln Ile Glu Leu Leu Glu Val Leu Thr Gly Phe Glu
85 90 95

Thr Asn Asn Lys Tyr Glu Ile Lys Asn Ser Phe Gly Gln Arg Val Tyr
100 105 110

Phe Ala Ala Glu Asp Thr Asp Cys Cys Thr Arg Asn Cys Cys Gly Pro
115 120 125

Ser Arg Pro Phe Thr Leu Arg Ile Ile Asp Asn Met Gly Gln Glu Val
130 135 140

Ile Thr Leu Glu Arg Pro Leu Arg Cys Ser Ser Cys Cys Cys Pro Cys
145 150 155 160

Cys Leu Gln Glu Ile Glu Ile Gln Ala Pro Pro Gly Val Pro Ile Gly
165 170 175

Tyr Val Ile Gln Thr Trp His Pro Cys Leu Pro Lys
180 185

(2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 568 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

CAGCATTCCA AGGACCTCCA GGATATAGTG GCTACCCTGG GCCCCAGGTC AGCTACCCAC 60

CCCCACCAGC CGGCCATTCA GGTCCTGGCC CAGCTGGCTT TCCTGTCCCA AATCAGCCAG 120

TGTATAATCA GCCAGTATAT AATCAGCCAG TTGGAGCTGC AGGGGTACCA TGGATGCCAG 180

CGCCACAGCC TCCATTAAAC TGTCCACCTG GATTAGAATA TTTAAGTCAG ATAGATCAGA 240

TACTGATTCA TCAGCAAATT GAACTTCTGG AAGTTTTAAC AGGTTTTGAA ACTAATAACA 300

AATATGAAAT TAAGAACAGC TTTGGACAGA GGGTTTACTT TGCAGCGGAA GATACTGATT 360

GCTGTACCCG AAATTGCTGT GGGCCATCTA GACCTTTTAC CTTGAGGATT ATTGATAATA 420

TGGGTCAAGA AGTCATAACT CTGGAGAGAC CACTAAGATG TAGCAGCTGT TGTTGTCCCT 480

GCTGCCTTCA GGAGATAGAA ATCCAAGCTC CTCCTGGTGT ACCAATAGGT TATGTTATTC 540

AGACTTGGCA CCCATGTCTA CCAAAGTT 568

(2) INFORMATION FOR SEQ ID NO:3:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 1266 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: double
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

CGTGTTGTGT ACGTAAGATT CAGGAAACGA AACCAGGAGC CGCGGGTGTT GGCGCAAAGG 60

TTACTCCCAG ACCCTTTTCC GGCTGACTTC TGAGAAGGTT GCGCAGCAGC TGTGCCCGAC 120

AGTCTAGAGG CGCAGAAGAG GAAGCCATCG CCTGGCCCCG GCTCTCTGGA CCTTGTCTCG 180

CTCGGGAGCG GAAACAGCGG CAGCCAGAGA ACTGTTTTAA TCATGGACAA ACAAAACTCA 240

CAGATGAATG CTTCTCACCC GGAAACAAAC TTGCCAGTTG GGTATCCTCC TCAGTATCCA 300

CCGACAGCAT TCCAAGGACC TCCAGGATAT AGTGGCTACC CTGGGCCCCA GGTCAGCTAC 360

CCACCCCCAC CAGCCGGCCA TTCAGGTCCT GGCCCAGCTG GCTTTCCTGT CCCAAATCAG 420

CCAGTGTATA ATCAGCCAGT ATATAATCAG CCAGTTGGAG CTGCAGGGGT ACCATGGATG 480

CCAGCGCCAC AGCCTCCATT AAACTGTCCA CCTGGATTAG AATATTTAAG TCAGATAGAT 540

CAGATACTGA TTCATCAGCA AATTGAACTT CTGGAAGTTT TAACAGGTTT TGAAACTAAT 600

AACAAATATG AAATTAAGAA CAGCTTTGGA CAGAGGGTTT ACTTTGCAGC GGAAGATACT 660

GATTGCTGTA CCCGAAATTG CTGTGGGCCA TCTAGACCTT TTACCTTGAG GATTATTGAT 720

AATATGGGTC AAGAAGTCAT AACTCTGGAG AGACCACTAA GATGTAGCAG CTGTTGTTGT 780

CCCTGCTGCC TTCAGGAGAT AGAAATCCAA GCTCCTCCTG GTGTACCAAT AGGTTATGTT 840

ATTCAGACTT GGCACCCATG TCTACCAAAG TTTACAATTC AAAATGAGAA AAGAGAGGAT 900

GTACTAAAAA TAAGTGGTCC ATGTGTTGTG TGCAGCTGTT GTGGAGATGT TGATTTTGAG 960

ATTAAATCTC TTGATGAACA GTGTGTGGTT GGCAAAATTT CCAAGCACTG GACTGGAATT 1020

TTGAGAGAGG CATTTACAGA CGCTGATAAC TTTGGAATCC AGTTCCCTTT AGACCTTGAT 1080

GTTAAAATGA AAGCTGTAAT GATTGGTGCC TGTTTCCTCA TTGACTTCAT GTTTTTTGAA 1140

AGCACTGGCA GCCAGGAACA AAAATCAGGA GTGTGGTAGT GGATTAGTGA AAGTCTCCTC 1200

AGGAAATCTG AAGTCTGTAT ATTGATTGAG ACTATCTAAA CTCATACCTG TATGAATTAA 1260

GCTGTA 1266

(2) INFORMATION FOR SEQ ID NO:4:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 422 amino acids
(B) TYPE: amino acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: peptide

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

Arg Val Val Tyr Val Arg Phe Arg Lys Arg Asn Gln Glu Pro Arg Val
1 5 10 15

Leu Ala Gln Arg Leu Leu Pro Asp Pro Phe Pro Ala Asp Phe Xaa Glu
20 25 30

Gly Cys Ala Ala Ala Val Pro Asp Ser Leu Glu Ala Gln Lys Arg Lys
35 40 45

Pro Ser Pro Gly Pro Gly Ser Leu Asp Leu Val Ser Leu Gly Ser Gly
50 55 60

Asn Ser Gly Ser Gln Arg Thr Val Leu Ile Met Asp Lys Gln Asn Ser
65 70 75 80

Gln Met Asn Ala Ser His Pro Glu Thr Asn Leu Pro Val Gly Tyr Pro
85 90 95

Pro Gln Tyr Pro Pro Thr Ala Phe Gln Gly Pro Pro Gly Tyr Ser Gly
100 105 110

Tyr Pro Gly Pro Gln Val Ser Tyr Pro Pro Pro Pro Ala Gly His Ser
115 120 125

Gly Pro Gly Pro Ala Gly Phe Pro Val Pro Asn Gln Pro Val Tyr Asn
130 135 140

Gln Pro Val Tyr Asn Gln Pro Val Gly Ala Ala Gly Val Pro Trp Met
145 150 155 160

Pro Ala Pro Gln Pro Pro Leu Asn Cys Pro Pro Gly Leu Glu Tyr Leu
165 170 175

Ser Gln Ile Asp Gln Ile Leu Ile His Gln Gln Ile Glu Leu Leu Glu
180 185 190

Val Leu Thr Gly Phe Glu Thr Asn Asn Lys Tyr Glu Ile Lys Asn Ser
195 200 205

Phe Gly Gln Arg Val Tyr Phe Ala Ala Glu Asp Thr Asp Cys Cys Thr
210 215 220

Arg Asn Cys Cys Gly Pro Ser Arg Pro Phe Thr Leu Arg Ile Ile Asp
225 230 235 240

Asn Met Gly Gln Glu Val Ile Thr Leu Glu Arg Pro Leu Arg Cys Ser
245 250 255

Ser Cys Cys Cys Pro Cys Cys Leu Gln Glu Ile Glu Ile Gln Ala Pro
260 265 270

Pro Gly Val Pro Ile Gly Tyr Val Ile Gln Thr Trp His Pro Cys Leu
275 280 285

Pro Lys Phe Thr Ile Gln Asn Glu Lys Arg Glu Asp Val Leu Lys Ile
290 295 300

Ser Gly Pro Cys Val Val Cys Ser Cys Cys Gly Asp Val Asp Phe Glu
305 310 315 320

Ile Lys Ser Leu Asp Glu Gln Cys Val Val Gly Lys Ile Ser Lys His
325 330 335

Trp Thr Gly Ile Leu Arg Glu Ala Phe Thr Asp Ala Asp Asn Phe Gly
340 345 350

Ile Gln Phe Pro Leu Asp Leu Asp Val Lys Met Lys Ala Val Met Ile
355 360 365

Gly Ala Cys Phe Leu Ile Asp Phe Met Phe Phe Glu Ser Thr Gly Ser
370 375 380

Gln Glu Gln Lys Ser Gly Val Trp Xaa Trp Ile Ser Glu Ser Leu Leu
385 390 395 400

Arg Lys Ser Glu Val Cys Ile Leu Ile Glu Thr Ile Xaa Thr His Thr
405 410 415

Cys Met Asn Xaa Ala Val
420

CLAIMS

We claim:

1. A preparation of a phospholipid scramblase, wherein the protein is approximately 35-37 kD as measured on a 12.5% SDS-polyacrylamide gel under reducing conditions.

2. The preparation of claim 1, wherein the phospholipid scramblase comprises residues 58 - 90 of SEQ ID NO:1.

3. The preparation of claim 1, wherein the phospholipid scramblase comprises residues 1 - 188 of SEQ ID NO:1.

4. The preparation of claim 1, wherein the phospholipid scramblase comprises residues 75-392 of SEQ ID NO:4.

5. The preparation of claim 1 wherein the protein is isolated from erythrocyte membranes.

6. The preparation of claim 1 wherein the protein is produced by cells genetically modified so as to express the protein of claim 1.

7. The preparation of claim 1 wherein the protein is produced by an organism selected from the group consisting of bacteria, insect cells, or yeast.

8. A recombinant DNA sequence encoding the protein of claim 1.

9. The sequence of claim 8, comprising SEQ ID NO:3.

10. The sequence of claim 8, comprising nucleotides 223-1176 of SEQ ID NO:3.

11. The sequence of claim 8, comprising SEQ ID NO:2 and wherein the sequence is part of a protein expression vector.

12. An inhibitor of phospholipid scramblase activity of the protein of claim 1.

13. The inhibitor of claim 12 wherein the inhibitor is an antisense nucleotide derived from a DNA sequence encoding P37.

14. The inhibitor of claim 12 wherein the inhibitor is an antisense nucleotide derived from a DNA sequence identified at SEQ ID NO:3.

15. The inhibitor of claim 12 wherein the inhibitor is a monoclonal antibody against P37.

16. The inhibitor of claim 12 wherein the inhibitor is a monoclonal antibody against peptide sequence identified as residues 385-392 of SEQ ID NO:4.

17. The inhibitor of claim 15 wherein the monoclonal antibody is selected from the group consisting of recombinantly produced intact IgG, Ig Fab fragment, recombinant Ig Fv or recombinant single chain Ig Fv fragment ScFv.

18. The inhibitor of claim 12 wherein the inhibitor is a peptide containing an amino acid sequence derived from P37, wherein the peptide is a competitive inhibitor of P37 phospholipid scramblase activity.

19. The inhibitor of claim 18 wherein the inhibitor is a peptide that binds to plasma membrane phosphatidylserine.

20. The inhibitor of claim 18 wherein the inhibitor is a peptide that prevents binding of Ca^{2+} to P37.

21. The inhibitor of claim 18 wherein the inhibitor prevents phosphorylation of P37 by a cellular protein kinase.

22. The inhibitor of claim 21 containing the peptide sequence Thr-Leu-Arg or is a peptidomimetic structural analogue of the tripeptide Thr-Leu-Arg.

23. A method for preventing the surface exposure of plasma membrane phosphatidylserine, phosphatidylethanolamine and cardiolipin on the surface of *in vitro* stored platelets or red blood cells comprising the step of adding an inhibitor of P37 to the stored platelets or red blood cells.

24. The method of claim 23 wherein the inhibitor is a nucleotide sequence.

25. The method of claim 23 wherein the inhibitor is a monoclonal antibody against P37.

26. The method of claim 23 wherein the inhibitor is a peptide containing an amino acid sequence derived from P37, wherein the peptide is an antagonist of P37 phospholipid scramblase activity.

27. The method of claim 26 wherein the inhibitor is a peptide that binds to plasma membrane phosphatidylserine.

28. The method of claim 26 wherein the inhibitor is a peptide that prevents binding of Ca^{2+} to P37.

29. The method of claim 26 wherein the inhibitor is a peptide or peptidomimetic peptide analogue that prevents phosphorylation of P37 by a cellular kinase.

30. A method for prolonging graft survival of transplanted organs and grafts comprising the step of adding an inhibitor of P37 phospholipid scramblase activity to an organ perfusate during *in vitro* organ storage.

31. A method for preventing the procoagulant properties of erythrocytes in sickle cell disease comprising the step of inhibiting erythrocyte P37 in a sickle cell patient.

32. A method for prolonging the *in vivo* survival of circulating blood cells comprising the step of preventing surface exposure of plasma membrane phosphatidylserine on the circulating blood cells by exposing the blood cells to an inhibitor of plasma membrane P37.

33. A method for treating autoimmune, thrombotic, thromboemolic, and inflammatory diseases comprising the step of treating a patient with an inhibitor of the phospholipid scramblase activity of P37.

34. The method of claim 33, wherein the disease is selected from disseminated intravascular coagulation, vascular thrombosis, fibrin generation during cardiopulmonary bypass procedures, rheumatoid arthritis, systemic lupus erythematosus, thrombotic thrombocytopenic purpura, heparin-associated thrombosis, and organ transplant rejection.

35. A method for diagnosing individuals with reduced or elevated capacity for platelet-promoted or erythrocyte-promoted fibrin clot activity comprising the step of quantitating the level of cellular expression of P37 in platelets and erythrocytes in a whole blood sample obtained from the patient mixed with acid-citrate-dextrose, EDTA, heparin, or other suitable anticoagulant.

36. The method of claim 35 wherein quantitation is performed by immunoblotting using an antibody to P37.

37. The method of claim 35 wherein quantitation is performed by ELISA assay using an antibody to P37.

38. The method of claim 35 wherein quantitation is performed by fluorescence-activated flow cytometry using an antibody to P37.

39. The method of claim 38 wherein quantitation is performed using a monoclonal antibody reactive with the peptide sequence corresponding to residues 385-392 of SEQ ID NO:4.

40. The method of claim 35 wherein quantitation is performed using oligonucleotides derived from P37 cDNA and the polymerase chain reaction.

41. The method of claim 35 wherein quantitation is performed by isolating P37 from a patient venous blood sample, by first isolating the platelets and erythrocytes by differential centrifugation, and then measuring the amount of P37 present either per cell or per unit of total cell protein, and comparing the measurement with control sample.

42. A genetically engineered cell for transplantation into a human or animal wherein the cell does not express PL scramblase at native levels of expression.

43. The cell of claim 42 further comprising a nucleotide molecule which is expressed by the cell and which codes for protein inhibiting the activity of PL scramblase.

44. The cell of claim 42 wherein the promoter of the PL scramblase gene is altered to either increase or decrease the expression of the gene.

45. The cell of claim 42 selected from the group consisting of endothelial cells, fibroblasts, epithelial cells, skeletal cells, cardiac and smooth muscle cells, hepatocytes, pancreatic islet cells, bone marrow cells, astrocytes, and Schwann cells

46. A prothesis for implantation in an animal or human having cells of claim 42 attached thereto.

47. The prosthesis of claim 46, wherein the prothesis is a vascular graft.

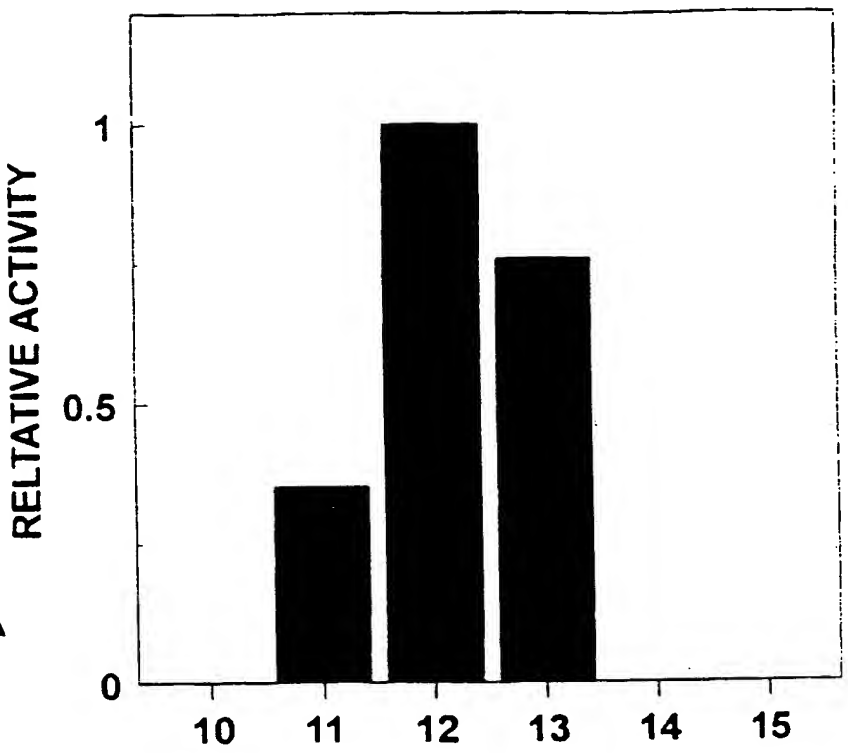


FIG 1A

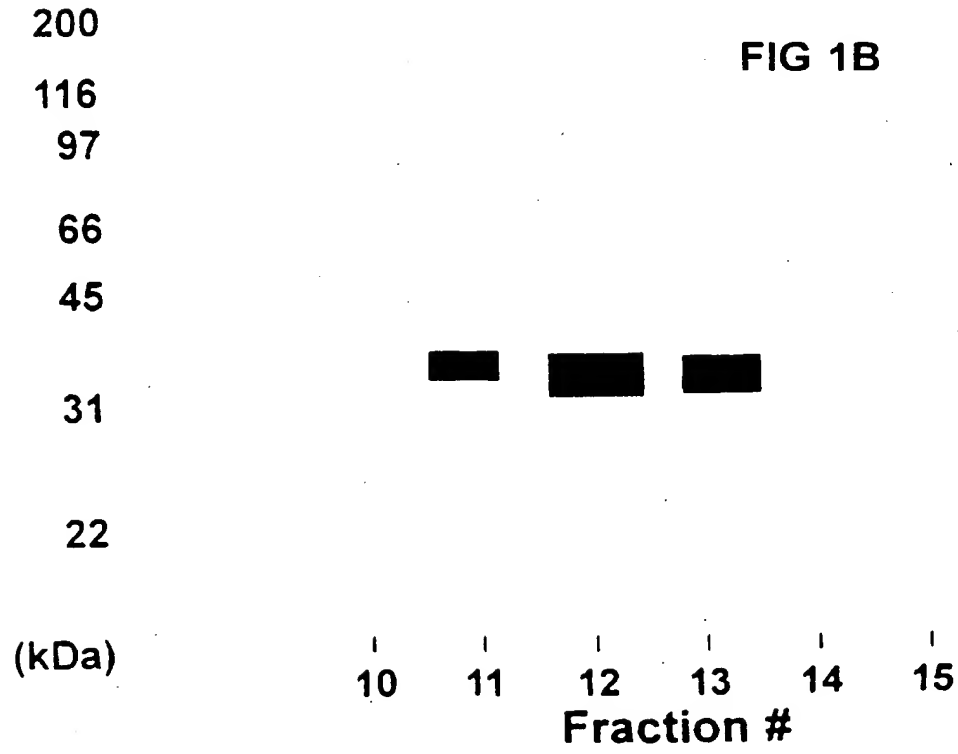


FIG 1B

FIG 2

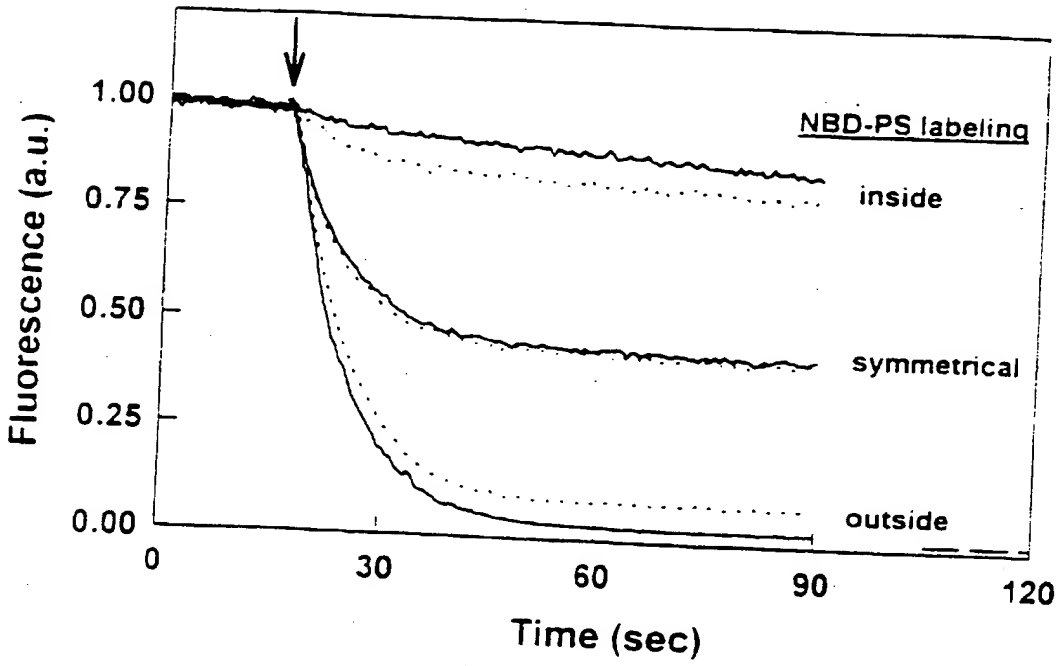


FIG 3

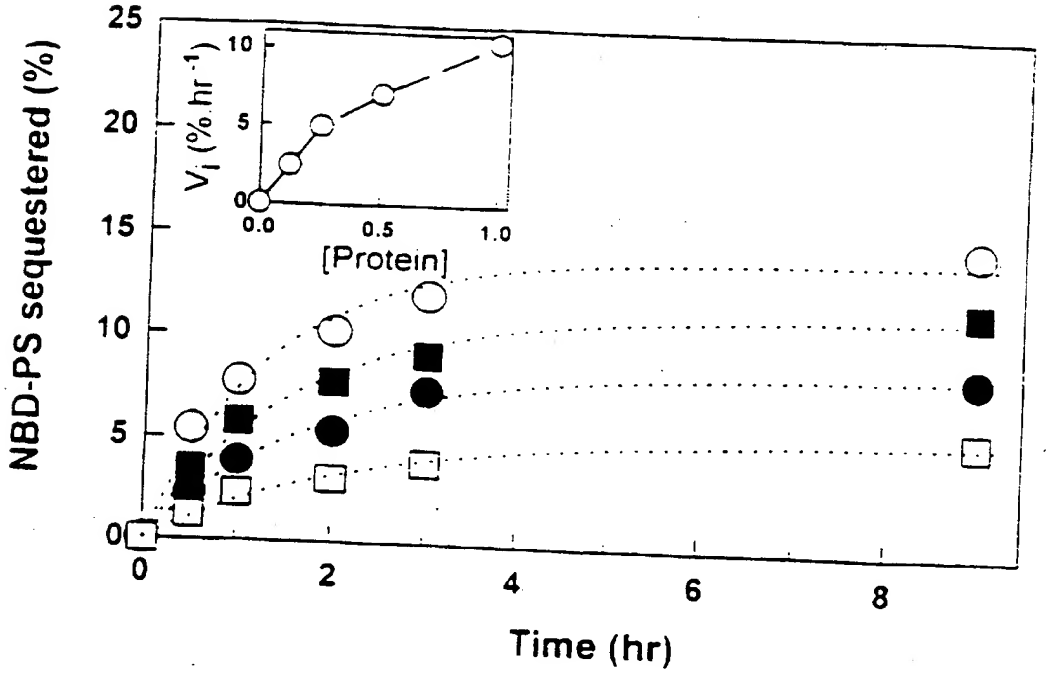
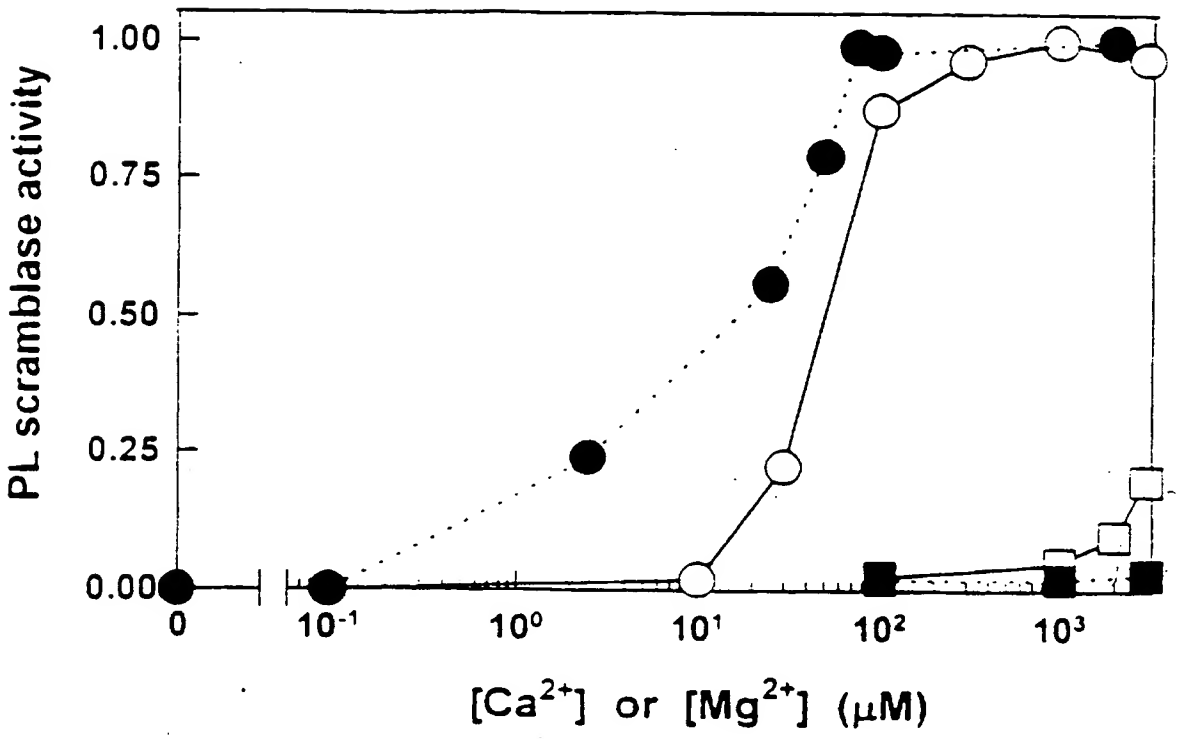


FIG 4


PL scramblase activity
1.00
0.75
0.50
0.25
0.00
0
10^{-1}
10^{0}
10^{1}
10^{2}
10^{3}
[Ca^{2+}] or [Mg^{2+}] (μM)

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US97/05198

A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) :G01N 33/573; C12N 9/00; C07K 16/00; C07H 21/04
US CL :435/6, 7.4, 13, 183; 530/388.15; 536/23.5
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 435/6, 7.4, 13, 15, 69.1, 183; 530/350, 388.15, 388.26; 536/23.5
424/9.1, 422, 146.1

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

APS, MEDLINE, BIOSIS, EMBASE, CAPLUS, WPIDS, CJACS
Sequence Databases (GENBANK, EMBL, PIR, SWISSPROT, EST, GENESEQ)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X,P ---- Y,P	Database, MPSRCH, SMITH-WATERMAN algorithm. Databases n-geneseq26 and EST-STS. May 1997, relevant hits, see entire document.	1-11 ---- 12-31, 33-47
X,P ---- Y,P	BASSE et al. Isolation of an Erythrocyte Membrane Protein that Mediates Ca^{2+}-dependent Transbilayer Movement of Phospholipid. J. Biol. Chem. 19 July 1996, Vol. 271, No. 29, pages 17205-17210. See entire document.	1-11 ---- 12-31, 33-47
X,P ---- Y,P	COMFURIUS et al. Reconstitution of Phospholipid Scramblase Activity from Human Blood Platelets. Biochem. 18 June 1996, Vol. 35, No. 24, pages 7631-7634. See entire document.	1-11 ---- 12-31, 33-47

[X] Further documents are listed in the continuation of Box C. [ ] See patent family annex.

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

Date of the actual completion of the international search
26 MAY 1997

Date of mailing of the international search report
11 JUL 1997

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Form PCT/ISA/210 (second sheet)(July 1992)*

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US97/05198

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	KOJIMA et al. Production and Characterization of Transformed B-Lymphocytes Expressing the Membrane Defect of Scott Syndrome. J. Clin. Invest. December 1994, Vol. 94, pages 2237-2244. See entire document.	1-29
Y	SMEETS et al. Calcium-induced transbilayer scrambling of fluorescent phospholipid analogs in platelets and erythrocytes. Biochem. Biophys. Acta. 02 November 1994, Vol. 1195, pages 281-286. See entire document.	1-29
Y	VERHOVEN et al. Mechanisms of Phosphatidylserine Exposure, A Phagocyte Recognition Signal, on Apoptotic T Lymphocytes. J. Exp. Med. November 1995, Vol. 182, pages 1597-1601. See entire document.	12-30, 33-34
Y	DEVAUX et al. Maintenance and consequences of membrane phospholipid asymmetry. Chem. Phys. Lipids. 06 September 1994, Vol. 73, pages 107-120. See entire document.	23-29
Y	Database MEDLINE on STN, No. 90158550, ZWAAL et al. Loss of membrane phospholipid asymmetry during activation of blood platelets and sickled cells; mechanisms and physiological significance. Mol. Cell. Biochem. 23 November-23 December 1989, Vol. 1-2, pages 23-31. Abstract.	31

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US97/05198

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

Please See Extra Sheet.

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. ☒ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:
1-31 and 33-47

4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest.

☐ No protest accompanied the payment of additional search fees.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US97/05198

BOX II. OBSERVATIONS WHERE UNITY OF INVENTION WAS LACKING
This ISA found multiple inventions as follows:

This application contains the following inventions or groups of inventions which are not so linked as to form a single inventive concept under PCT Rule 13.1.
Group I, claim(s) 1-11, drawn to phospholipid scramblase protein and DNA encoding same.
Group II, claim(s) 12-22, drawn to inhibitors of scramblases.
Group III, claim(s) 23-29, drawn to uses of inhibitors to prevent surface exposure of phospholipids in stored red blood cells or platelets.
Group IV, claim(s) 30, drawn to uses of inhibitors to prolong graft survival.
Group V, claim(s) 31, drawn to uses of inhibitors to prevent coagulation of cells in a sickle cell patient.
Group VI, claim(s) 32, drawn to uses of inhibitors to prolong red blood cell lifespan in vivo.
Group VII, claim(s) 33-34, drawn to uses of inhibitors to treat autoimmune diseases.
Group VIII, claim(s) 35-41, drawn to methods of diagnosing patients with abnormal capacities for clot formation.
Group IX, claim(s) 42-47, drawn to methods of engineering cells for transplantation.

The inventions listed as Groups I-IX do not relate to a single inventive concept under PCT Rule 13.1 because, under PCT Rule 13.2, they lack the same or corresponding special technical features for the following reasons: Group I and Group II are drawn to separate products that do not necessarily have anything in common, either structurally or functionally. For example, the scramblases of Group I are Ca++ dependent, so they would be inhibited by any compound that chelates divalent cations, such as EDTA or citric acid. Groups III-VII are drawn to five separate uses of the inhibitors. Group VIII includes methods of diagnosis that do not necessarily use the first named product (they might instead use antibodies or coagulation tests). Group IX includes methods of engineering cells that do not necessarily involve any direct changes in the compounds of group I, such as the addition of nucleic acids coding for an inhibitor.